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Properties of a conductive cellular chloride pathway in the skin of the toad (*Bufo bufo*)

By

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Abstract

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Two types of chloride current response to step-wise hyperpolarization of the toad skin is demonstrated: (1) An "instantaneous" response observed immediately upon voltage change, and (2) subsequent slow response, the time course of which is sigmoidal. The slow response is due to an increase of a transcellular conductance which is specific to chloride ions. The time constant of the conductance increase is dependent on the amplitude of the transepithelial voltage displacement, the smallest time constants are obtained for the highest potentials and are in the order of 30 s. The voltage dependence of the steady-state conductance and the steady-state chloride current reveal that the chloride pathway has maximum conductance for $V = 80$ mV (outside of the skin being negative) and approaches non-conducting state for $V > 0$ mV. This strong outward going rectification is a steady-state phenomenon. In skins hyperpolarized for few minutes, the "instantaneous" I-V curves show that the chloride pathway in the conducting state allows large inward chloride current (outward chloride flux) to pass in the voltage range $40 \text{ mV} > V > 0$ mV. Calculations based on a three-compartment model indicate that the strong steady-state chloride current rectification cannot be explained if only the intracellular chloride concentration and the membrane potential are allowed to vary ("Goldman-rectification"). It is suggested, therefore, that the permeability of the chloride pathway varies reversibly with the transepithelial potential difference. The variable which controls the chloride permeability may be apical/membrane potential or the concentration of an intracellular ion.

In previous paper (Bruus, Kristensen and Hvid Larsen 1976) it was shown that the chloride transport across the skin of the toad *Bufo bufo* is predominantly transcellular. In the electrically short circuited preparation, two different cellular chloride pathways could be distinguished. An exchange diffusion and a rheogenic active transport directed towards the inside. We could furthermore show that at clamping voltages larger than or equal to the spontaneous transepithelial electrical potential difference, an acetazolamide (Diamox) sensitive, charge transferring inward transport of chloride exists. Due to this transcellular conductive chloride pathway the spontaneous potential difference across this preparation is only about 20 mV (outside being negative), provided both sides are bathed with NaCl-Ringer's solution.

Analysis of the voltage dependence of unidirectional chloride fluxes revealed that the

steady state chloride net flux (chloride current) is not a linear function of clamping voltage. The present paper deals with a closer examination of this voltage dependent behaviour. By means of transepithelial voltage clamps, the time course of chloride current activation is described and the steady-state voltage conductance relation obtained. The time dependent behaviour and the non-linear steady-state properties of the chloride pathway may originate from changes in intracellular ion concentrations and from changes in membrane potentials of the transporting cells. In order to estimate the significance of such changes for the steady-state current voltage relation, a three-compartment model based on the two-membrane hypothesis is developed for quantitative analysis.

The subsequent paper deals with the relation between the exchange pathway and the conductive pathway for chloride transport in toad skin.

The results presented below have been reported briefly (Hvid Larsen and Kristensen 1976).

Methods

Animals and composition of Ringer solutions Isolated abdominal skins of the toad *Bufo bufo* (L.) in the middle of the intermolt phase were used throughout this study. Handling of animals in the laboratory prior to experimentation has been described previously (Brown *et al.* 1976). In the present experiments the skins were always bathed with *NaCl-Ringer*s on the inside, the composition of which is (mmole/liter) Na⁺ 114.0, K⁺ 4, Ca⁺⁺ 1.0, Cl⁻ 114.0, gluconate = 2.0, HCO₃⁻ ~ 4, and pH = 8.2. The outside medium and will be referred to as *NaCl-Ringer*, *Na-gluconate-Ringer* (chloride substituted by gluconate), *KCl-Ringer* (sodium substituted by potassium) and *K-gluconate-Ringer* (sodium and chloride substituted by potassium and gluconate, respectively). The experiments were performed at room temperature (20–22 °C).

Experimental set up For isotope flux measurements, conical lucite chambers with an exposed area of 7 cm² were used (Koeleff-Johnsen, Ussal and Zerahn 1952). However many of the experiments presented below were performed by means of a lucite chamber device, similar to the one used by Whittembury (1964). The outer chamber forms an open cup, whose bottom is a 2 cm exposed skin area resting on a nylon net and tied with a silk thread onto the lucite cylinder which forms the inner chamber. The volume of the solution in the outer chamber is 1 ml and the outside of the skin can be perfused continuously with Ringer's at a flow rate of 2 ml s⁻¹. By manually operating an eight-way stopcock, the perfusion solution can be selected from 8 different flasks. The performance of this set-up was tested by replacing the skin by an anion-selective membrane (Series 100 Amicon). The half time of the potential change after increasing the outside chloride concentration from 10 to 100 mM was found to be 1 s. (In this calculation, the logarithmic relation between transmembrane potential and concentration was taken into account, cf. Dainty and Hoose 1966). This response is not a very fast one and it indicates that a considerable unstirred layer affects the potential response of the anion selective membrane. However with toad skin mounted, the response of the short circuit current to sudden removal of outside sodium has considerably longer half times which vary from 5 to 18 s (see Fig. 4B). Applying the very same procedure with frog skin (*Rana temporaria*) mounted in the chamber leads to half time values for short circuit current decrease of 17 s. This indicates that the toad skin preparation, barrier outside, the sodium selective membrane prevents very fast exposure of this membrane to the outer bulk solution. This assumption agrees well with the finding that amiloride added to the outer solution blocks the active sodium transport rather slowly with a half time of the decrease in short circuit current of about one minute. This value is 30 times higher than that observed with the frog skin preparation mounted in the same chamber. In view of this finding we did not attempt to improve upon the perfusion conditions.

Voltage clamping For voltage clamping, conventional circuits were used with open loop gains of 1000 and with a rise time of step trans-epithelial voltage displacements of either 100 ms or 8 ms. For potential measurements, calomel electrodes (K401 Radiometer Copenhagen) supplied with 3 M-saturated KCl bridges were used. The tips of the bridges were placed close to the center of the skin. As a rule, two Ag/AgCl half cells supplied current through the skin. In some experiments, however, ring shaped platinum electrodes replaced the Ag/AgCl half cell of the outer chamber. The current electrodes were placed in such a way to secure a uniform current density through the exposed skin area.

3 types of voltage clamp studies were performed. In the first, the skin was allowed to reach the steady-state open circuit potential, the circuit was then switched to and maintained in the voltage clamp mode for a period of time sufficient to obtain steady clamping current. In the second type, the procedure was identical with the first except for superpositioning of square shaped voltage goal of either 20 mV or 50 mV and of 500 ms duration supplied intermittently from the circuit. From the current responses to these voltage displacements, the time course of the skin conductance following voltage clamping as calculated in the third type of voltage clamp study the skin as clamped at predicted holding potential. When steady clamping current was obtained, a staircase generator was triggered manually which fed staircase shaped voltage goal to the command voltage input of the clamp circuit. 10-15 steps of 20 or 10 mV each and of 50 ms duration were used.

The time course of clamping current following voltage clamping was so slow that conventional potentiometer (R.E. 511 or R.E. 520 Servogor) was sufficient to monitor the current response. A higher time resolution was needed to record the current responses to the voltage staircase. For this purpose, transient recorder (DL 901 data-b) and storage oscilloscope (R 5031 Tektronix) are used.

Isotope flux measurements. Steady-state transepithelial fluxes of chloride were measured with ^{36}Cl (Radiochemicals) as described by Brown *et al.* 1976.

Ammonia as ammonia HCl was supplied from Merck, Sharp & Dohme.

Notes on the leak conductance. At a rather late stage of this study it was realized that the leak conductance in preparations mounted in the perfusion chamber was significantly higher than the previously reported very low paracellular sheet conductance. This difference may be conveniently illustrated by comparing the total skin conductance in preparations mounted in the two types of chambers and exposed to K-glucosate-Ringer outside. In the flux chamber, the conductance values ranged from 0.01 to 0.09 mS/cm² whereas preparations mounted in the perfusion chamber gave 0.1-0.5 mS/cm². The first mentioned values are close to the previously estimated $G_{\text{leak}} = 0.074$ mS/cm² calculated from isotope flux measurements (Brown *et al.* 1976), and indicates that the skin conductance obtained with K-glucosate Ringer outside gives reasonably good estimate of the paracellular sheet conductance. (A similar method for quick estimate of the paracellular conductance as used by Ussing and Windhager (1964), using $\text{K}_2\text{S}_2\text{O}_8$ -Ringer instead of K-glucosate Ringer.) The conductances derived from the experiments below however are not affected by the presence of larger leak conductance since in both types of preparations the leak pathway has linear current-voltage characteristics with time-invariant conductance in the range $-100 \text{ mV} < V < 50 \text{ mV}$.

Sign convention. When dealing with epithelia, the most widely used current sign notation is to define an ion as going current positive. Consequently the transepithelial potential difference (V) should be defined according to $V = \psi_o - \psi_i$, here ψ_o and ψ_i are the potentials of the outer and inner bathing solutions, respectively. These sign notations are used in the present and in the subsequent paper. It is further noted that "indepolarization" is used in the sense of increasing the negativity of the outside potential relative to the inside potential.

The quantitative analysis of the three-compartmental model as performed on Digital Equipment PDP/6.

Results

Non-steady-state current response to voltage clamp

The response of transepithelial current to stepwise changes in transepithelial voltage from the resting value to a predetermined value is shown in Fig. 1. The records were obtained as follows. The skin was equilibrated at open circuit conditions until a steady resting potential was achieved. The voltage was then stepped to the desired value, and the time course of the clamping current was recorded. After a 5-10 min period of voltage clamping, the skin was again allowed to relax at open circuit conditions for a period of time sufficient to bring the spontaneous potential difference back to its initial resting value. The procedure was repeated for the 5 subsequent clamping voltages indicated. In the example shown in Fig. 1 the steady-state resting potential between the sequential voltage clamping periods, was -22 to -24 mV. In other words, the non-steady-state phenomena observed upon voltage clamping are reversible.

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The subsequent paper deals with the relation between the exchange pathway and the conductive pathway for chloride transport in toad skin.

The results presented below have been reported briefly (Hvid Larsen and Kristensen 1976).

Methods

Animals and composition of Ringer solutions. Isolated abdominal skins of the toad *Bufo bufo* (L.) in the middle of the hibernation phase were used throughout this study. Handling of animals in the laboratory prior to experimentation has been described previously (Brus *et al.* 1976). In the present experiments the skins were always bathed with a *Cl*-Ringer on the inside, the composition of which is (mmole/liter): $\text{Na}^+ 114.6$, $\text{K}^+ 2.4$, $\text{Ca}^{++} 1.0$, $\text{Cl}^- 114.0$, gluconate ~ 0 , $\text{HCO}_3^- 2.4$, d.p.H. ~ 8.2 . The outside medium used and will be referred to as *NaCl-Ringer*, *Na-gluconate-Ringer* (chloride substituted by gluconate), *KCl-Ringer* (sodium substituted by potassium), and *K-gluconate-Ringer* (sodium and chloride substituted by potassium and gluconate, respectively). The experiments were performed at room temperature (20–22 °C).

Experimental set-up. For short-circuit flux measurements, conical lucite chambers with an exposed area of 7 cm² were used (Koeeford-Johnsen, Ussing and Zerahn 1952). However, many of the experiments presented below were performed by means of a lucite chamber device, similar to the one used by Whittembury (1964). The outer chamber forms an open cup whose bottom is a 2 cm² exposed skin area resting on a nylon net attached with silk thread to the lucite cylinder which forms the inner chamber. The volume of the solution in the outer chamber is 5 ml, and the outside of the skin can be perfused continuously with Ringer's at a flow rate of 2 ml/s. By manually operating a eight-way stopcock the perfusate solution can be selected from 8 different flasks. The performance of this set-up was tested by replacing the skin by an anion-selective membrane (Series 100 Amicon). The half time of the potential change after increasing the outside chloride concentration from 10 to 100 mM was found to be 1 s. (In this calculation, the logarithmic relation between transmembrane potential and concentration was taken into account, cf. Ussing and Høuse 1966.) This response is not a very fast one and it indicates that a considerable, unstirred layer affects the potential response of the anion selective membrane. However, with toad skin mounted the response of the short-circuit current to sudden removal of outside sodium has considerably longer half times which vary from 5 to 18 s (see Fig. 4 B). Applying the very same procedure used with frog skin (*Rana temporaria*) mounted in the chamber leads to half time values for short-circuit current decrease of 1.7. This indicates that, in the toad skin preparation, a barrier outside the sodium selective membrane prevents very fast exposure of this membrane to the outer bulk solution. This assumption agrees well with the finding that in toad added to the outer solution blocks the active sodium transport rather slowly, with half time of the decrease in short-circuit current of about one minute. This value is 30 times higher than that observed with the frog skin preparation mounted in the same chamber. In view of this finding we did not attempt to improve upon the perfusion conditions.

Voltage clamping. For voltage clamping, conventional circuits were used with open loop gain of 1000 and with rise time of step trans-epithelial voltage displacements of either 100 ms or 8 ms. For potential measurements, calomel electrodes (K401 Radiometer Copenhagen) supplied with 3 M agar-0.3 M KCl bridges were used. The tips of the bridges were placed close to the center of the skin. As a rule two Ag/AgCl half cells supplied current through the skin. In some experiments, however, a ring shaped platinum electrode replaced the Ag/AgCl half cell of the outer chamber. The current electrodes were placed such as to secure uniform current density through the exposed skin area.

3 types of voltage clamp studies are performed. In the first, the skin was allowed to reach the steady-state open circuit potential, the current was then switched to and maintained in the voltage clamp mode for a period of time sufficient to obtain steady clamping current. In the second type, the procedure is identical with the first except for superpositioning of square shaped voltage gain of either 20 mV or 50 mV and of 500 ms duration applied intermittently from the circuit. From the current responses to these voltage displacements, the time course of the skin conductance following voltage clamping was calculated. In the third type of voltage clamp study the skin is clamped at predicted holding potential. When steady clamping current was obtained, staircase generator was triggered manually back fed staircase shaped voltage signal to the constant voltage input of the clamp circuit. 10-15 steps of 20 or 10 mV each and of 50 ms duration were used.

The time course of clamping current following voltage clamping was so slow that conventional pen-recorder (RE 511 or RE 520 Servogor) is sufficient to monitor the current response. A higher time resolution is needed to record the current responses to the voltage staircase. For this purpose, transient recorder (DL 901 datab) and storage oscilloscope (R 3031 Tektronix) are used.

Isotope flux measurements. Steady-state transepithelial fluxes of chloride were measured with ^{36}Cl (Rise Denmark) as described by Brown *et al.* (1976).

Analytical reagents. Anionomer HCl was supplied from Merck, Sharp & Dohme.

Note on the leak conductance. At earlier late steps of this study it is realized that the leak conductance in preparations mounted in the perfusion chamber is significantly higher than the previously reported very low paracellular shunt conductance. This difference may be conveniently illustrated by comparing the total skin conductance in preparations mounted in the two types of chambers and exposed to K-glucuronate Ringer outside. In the first chamber, the conductance values ranged from 0.05 to 0.09 mS/cm² whereas preparations mounted in the perfusion chamber gave 0.1-0.5 mS/cm². The first set of values are close to the previously estimated 1.5×10^{-4} mS/cm² calculated from isotope flux measurements (Brown *et al.* 1976), and indicates that the skin conductance obtained with K-glucuronate Ringer outside gives reasonably good estimate of the paracellular shunt conductance. (A similar method (or quick estimate of the paracellular conductance was used by Ussing and Windhager (1964), using K_2SO_4 -Ringer instead of K-glucuronate Ringer). The conclusions derived from the experiments below however are not affected by the presence of larger leak conductance since in both types of preparations the leak pathway has linear current-voltage characteristics with zero-current conductance in the range ~ 100 mV \sim V/50 mV.

Sign convention. When dealing with epithelia, the most widely used current sign notation is to define an inward going current positive. Consequently the transepithelial potential difference (V) should be defined according to $V = \psi_1 - \psi_2$ here ψ_1 and ψ_2 are the potentials of the outer and inner bathing solutions, respectively. These sign notations are used in the present and in the subsequent paper. It is further noted that "hyperpolarization" used in the sense of increasing the negativity of the outside potential relative to the inside potential.

The quantitative analysis of the three-compartment model was performed on Digital Equipment PDP11/4.

Results

Non-steady-state current response to voltage clamp

The response of transepithelial current to stepwise changes in transepithelial voltage from the resting value to a predetermined value is shown in Fig. 1. The records were obtained as follows. The skin was equilibrated at open circuit conditions until steady resting potential was achieved. The voltage was then stepped to the desired value, and the time course of the clamping current was recorded. After a 5-10 min period of voltage clamping, the skin was again allowed to relax at open circuit conditions for a period of time sufficient to bring the spontaneous potential difference back to its initial resting value. The procedure was repeated for the 5 subsequent clamping voltages indicated. In the example shown in Fig. 1 the steady-state resting potential between the sequential voltage clamping periods, was -22 to -24 mV. In other words, the non-steady-state phenomenon observed upon voltage clamping are reversible.

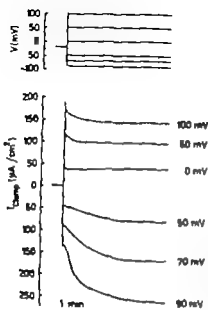


Fig. 1 Records of clamping current (I_{clamp}) following a step change in transepithelial potential difference (V). For each record V was changed from its steady-state spontaneous value to the clamping voltage indicated. Steady-state spontaneous value of $V = -22$ to -24 mV. The records were obtained from one toad skin preparation exposed to NaCl-Ringer's on both sides.

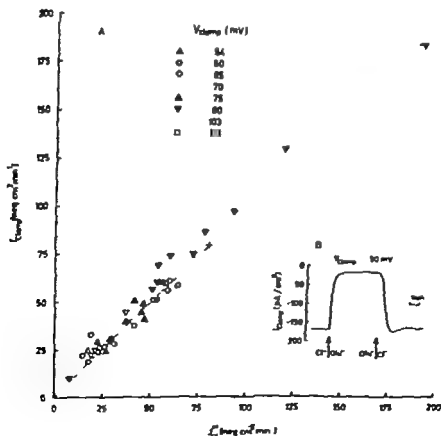
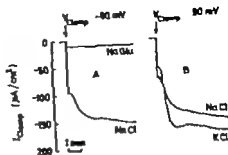


Fig. 2. A. Plot of steady-state outward clamping current (I_{clamp}) versus simultaneously measured steady-state chloride influx (J). Each point represents an average of two consecutive half hour periods at the clamping voltage indicated and with NaCl-Ringer's bathing both sides of the skin. Total number of skins 40. The dashed curve indicates the line of identity. B. Response of outward clamping current to substitution of outside chloride by gluconate. Half time of current decrease following removal of outside chl

Fig. 3. *A* Time course of clamping current following voltage clamp at -90 mV in the presence of chloride and gluconate outside, respectively. Note: with gluconate outside, I_{clamp} is time-invariant. *B* Same as in *A*, but with different cations outside. With Na^+ as well as with K^+ outside, I_{clamp} displays similar time-dependent behaviour.



Typically the outward current increases with time upon hyperpolarization and the inward current decreases with time for clamping voltages above zero mV. In neither case is the time course of the current response simple. For example, the half time of current change is dependent on voltage. Also note that the outward current shows a marked initial inflexion for large negative voltage values. The subsequent paragraphs of this paper deal with an investigation of the time-variant behaviour of the clamping current recorded during hyperpolarization.

Contribution of Cl^- -influx to outward clamping current

The relation between outward clamping current and inward movement of chloride ions was established by chloride isotope flux measurements. Fig. 2 *A* shows all simultaneous measurements of steady-state outward clamping currents and steady-state chloride influxes calculated from the inward movement of ^{36}Cl . It can be seen that at these voltages the influx of chloride closely matches the outward current flux, although the steady-state clamping currents obtained from different skin preparations vary by a factor of more than ten. This equivalence of clamping current and chloride influx in skins bathed on both sides with NaCl-Ringer's implies that at $V < -55$ mV the clamping current is governed by the conductance of a pathway selectively allowing chloride ions to pass. It is expected, therefore, that substitution of chloride by α -gluconate in the solution bathing the outside of the skin will significantly reduce the steady-state outward clamping current. Fig. 2 *B* shows that this is the case.

As a simple approach towards an identification of the ionic components involved in the non-steady-state response to voltage clamp, anion and cation substitutions in the outer bath were performed. Fig. 3 *A* shows the time course of clamping current recorded from skins exposed to chloride-Ringer's and to chloride-free-Ringer's outside. When the external chloride concentration is reduced to zero, the instantaneous outward current is reduced by one order of magnitude, and the time dependence of the outward current completely disappears. Substitution of sodium ions by potassium ions in the solution bathing the outside of the skin is expected to reduce cellular sodium activity (Korfoed-Johnsen and Ussing 1955). This treatment, however, does not affect the main characteristics of the outward current response to maintained hyperpolarization, cf. Fig. 3 *B*. These results strongly suggest that the time-variant behavior of the outward current is restricted to a pathway selectively passing chloride ions.

TABLE 1 Effect of amiloride on chloride influx and outward clamping current. Skins bathed in NaCl Ringer's on both sides. Amiloride added to the outer solution. All fluxes and current values are averages of two consecutive half hour periods and are expressed in $\text{nmol cm}^{-2} \text{ min}^{-1}$

Animal no	Spontaneous potential mV	$J_{\text{Cl}}^{\text{in}}$	Clamping potential mV	Control		5 μM amiloride	
				$J_{\text{Cl}}^{\text{in}}$	I_{clamp}	$J_{\text{Cl}}^{\text{in}}$	I_{clamp}
1	-14	14.9	-60	24.6	-21.3	22.2	-28.4
2	-13	14.3	-60	59.5	-52.9	58.5	-51.9
3	-11	12.9	-60	34.3	-31.1	29.0	-38.2
4	-14	16.7	-60	62.9	-64.8	69.6	-64.1

Effect of amiloride

Amiloride added to the solution bathing the outside of the amphibian skin inhibits sodium transport across the outer sodium selective membrane (Dörge and Nagel 1970, Cuthbert and Shum 1974, Morel and Leblanc 1975, Fuchs, Hvid Larsen and Lindemann 1977). As can be seen from Table 1 neither the steady-state chloride influx nor the steady-state clamping current observed at $V_{\text{clamp}} = -60$ mV are affected by exposing the skin to 5 μM amiloride outside. The insensitivity of inward chloride flux to amiloride was also demonstrated in another type of experiment the result of which is shown in Fig. 4 A. Substitution of chloride by gluconate in the solution bathing the outside of the skin reduces outward clamping current from $-188 \mu\text{A/cm}^2$ to $-20 \mu\text{A/cm}^2$. Neither the steady-state clamping current nor the decrease ($-I_{\text{Cl}}$) observed after reducing the outer chloride concentration to zero mM is significantly affected by the presence of 60 μM amiloride in the outer solution. This concentration of amiloride, however, abolishes the active sodium flux (Fig. 4 B). The short circuit current is almost entirely carried by sodium ions, as demonstrated by its reduction towards

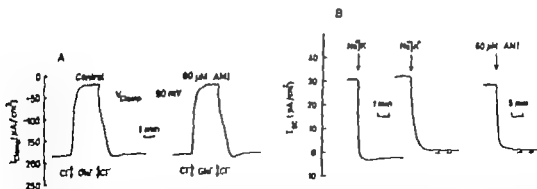


Fig. 4 A. Response of outward clamping current ($V_{\text{clamp}} = -90$ mV) to chloride-gluconate substitution in outside bathing solution. Left and right hand records were obtained from the same skin preparation bathed with NaCl-Ringer's on both sides, the latter however after 25 min. of exposure to 60 μM amiloride outside. Neither the amplitude of current decrease (I_{Cl}) nor the half time of current response ($T_{1/2}$) is affected by the presence of amiloride. $I_{\text{Cl}} = -168 \mu\text{A/cm}^2$, $T_{1/2} = 12$ s.

Fig. 4 B. Response of short circuit current (I_{sc}) upon Na⁺/K⁺ substitution in outside bathing solution. Two left hand record illustrate the difference of a "fast and a slow responding preparation" ($T_1 = 5$ and 18 s, respectively). Right hand panel shows the response of short circuit current to addition of 60 μM amiloride outside. Note the different time scale. $T_1 = 60$ s.

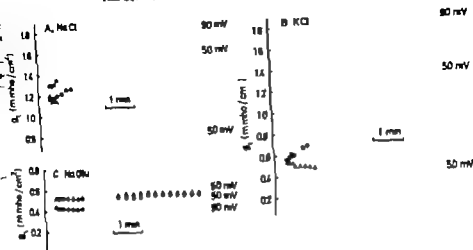


Fig. 5 Time course of total skin conductance (G_s) following step voltage clamp from the steady-state spontaneous potential difference. A NaCl-Ringer's outside. B KCl-Ringer's outside. C Na-glucuronate-Ringer's outside.

zero upon substitution of outside sodium by potassium. $60 \mu\text{M}$ amiloride almost completely inhibits this inward active sodium flux (right hand part of Fig. 4 B).

1 slope-conductance relations

The results presented in the preceding sections have shown that hyperpolarization of the skin brings about a stimulation of an outward current carried by an inward transport of chloride ions. In another series of expts. the time course of total skin conductance and the voltage dependence of the steady-state conductance were investigated. Like in the experiments presented above, the skin was pre-equilibrated under open circuit conditions, when a constant open circuit potential was achieved, the potential was changed in a step-wise fashion and maintained at the new value for several minutes. The clamping current was recorded and the skin conductance measured intermittently by recording the current response in a square voltage pulse of 20 or 50 mV amplitude and of 500 ms duration. These intermittent conductance measurements do not affect the overall current time course.

The time course of conductance change is illustrated by representative examples in Fig. 5. When exposed to NaCl- or KCl-Ringer's but not to Na-glucuronate Ringer's outside, the conductance is time variant upon a step change of transepithelial voltage. Especially for large negative values of V_{clamp} the time course of conductance change is clearly sigmoidal. Upon depolarization to $+50$ mV the conductance decreases in skins exposed to NaCl-Ringer's outside. This response is not typical with KCl-Ringer's outside, however with potassium outside, the spontaneous potential is about zero mV as compared to -25 mV with sodium outside.

The time it takes for the conductance to reach its new steady value is dependent on the amplitude of voltage change. In order to facilitate comparison of the rate of conductance increase for different step voltage changes, the time required to reach half maximal conductance change, $T_{1/2}$, will be used. Table 11 summarizes $T_{1/2}$ values obtained from above.

TABLE II T_1 values (for definition of T_1 see text) of conductance increase after step voltage change from spontaneous potential to a prespecified clamping potential.

Animal no	Spontaneous potential mV	T_1 , seconds				
		Clamping potential, mV ...	-90	-80	-70	-50 -30
1	-19 to -26		39	42	42	60 102
2	-24 to -27 ^a		48	53	55	66 83
	-2 ^b		50	54	53	70 90
3	-4 to -28 ^a		40	—	51	108 —
4	-22 to -23 ^a		60	—	95	120 —
5	-17 ^a		33	—	50	55 —
5	-2 ^c		28	—	60	120 —
6	-16 to -19 ^a		60	—	70	105 —
7	-27 to -28 ^a		40	—	80	90 —

^a NaCl Ringer's outside.

^b NaCl-Ringer's outside + 60 μ M amiloride.

^c KCl Ringer's outside.

different experimental conditions. Although the absolute values for equal voltage conditions vary from skin to skin, the general picture is quite uniform. T_1 ranges from about 30 to 120 s, the smallest values invariably are obtained for the largest command voltage steps.

Steady-state conductance values (g_{∞}^0) are reached between $t = 3$ min and $t = 5$ min, depending on voltage. In Fig. 6 the variation of steady state conductance with clamping voltage under different experimental conditions is depicted. In the following these curves are denoted g_{∞}^0 -V curves. The g_{∞}^0 -V curve for skins exposed to NaCl-Ringer's outside is S-shaped, with a maximum g_{∞}^0 at V well below the resting potential and the minimum above zero mV (Fig. 6 A and B). The main characteristics of the g_{∞}^0 -V curve is not affected by application of 60 μ M amiloride outside, or by substitution of outside sodium by potassium (Fig. 6 C and D). This gives strong support to the view that the sigmoidal g_{∞}^0 -V relation is not due to sodium channel processes, but is a characteristic feature of a chloride conducting pathway. The different g_{∞}^0 -V curve obtained with a chloride free outer solution supports this hypothesis (Fig. 6 F).

Oubain added to the solution bathing the inside of the frog skin reduces the active transepithelial sodium transport by inhibiting the active Na/K pump located at the inward facing membrane (Koefoed-Johnsen 1957; Dörge and Nagel 1972). Addition of ouabain to the skin likewise inhibits, although slowly, the active sodium transport. In the skin from which the results shown in Fig. 6 E are obtained, the short circuit current was reduced from 18 μ A/cm to 1 μ A/cm after 3 h of exposure to 10^{-6} M ouabain. However, even after this treatment the S-shaped g_{∞}^0 -V curve was retained.

Relation between steady state chloride current and transepithelial potential difference

Steady-state current values are depicted as a function of clamping voltage in Fig. 7 A, B and C. These steady-state I-V curves are non-linear and show outward going rectification, which can be deduced from the g_{∞}^0 -V relations discussed above. Blocking the sodium channels with amiloride interferes selectively with the transcellular sodium current. Another way of elimin-

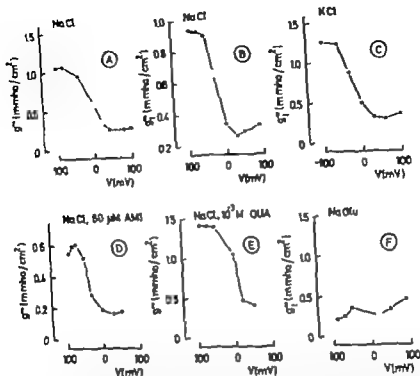


Fig. 6. Examples of steady-state total skin conductance (g^s) as a function of transepithelial clamping voltage (V). The outside Ringer's solutions are indicated for each curve. Results from the anion-free Ringer's solution were obtained 30 min after addition of the drug to the other solution. Ouabain was added to the latter bathing solution and the measurements performed after 3 h of drug exposure.

replacing the sodium current is to replace sodium by potassium in the solution bathing the outside of the skin. Thus, the $I-V$ curve obtained under either of these conditions comes close to that of the shunt pathway plus the cellular chloride conducting pathway. In general, the shunt pathway in toad skin exposed to chloride-Ringer's on both sides has linear $I-V$ characteristics for the voltage range investigated, $-80 \text{ mV} < V < +40 \text{ mV}$ (Bruss *et al.* 1976). Thus after inactivation of sodium channels we have:

$$I_{\text{clamp}} = I_{\text{Cl}} + V g_{\text{shunt}} \quad (1)$$

where I_{clamp} is the steady-state clamping current at voltage V , g_{shunt} is the shunt conductance, and I_{Cl} is the steady-state chloride current through the voltage dependent cellular pathway. For each skin, g_{shunt} may be approximated by the skin conductance obtained with K-glucuronate Ringer's outside (cf. p. 3). Taking I_{clamp} values from curves B and C of Fig. 7 and using the g_{shunt} estimated as described above, we have calculated the steady-state current voltage curve of the chloride conducting pathway (see Fig. 7 E and F). Another method of determining the $I_{\text{Cl}}-V$ relation was suggested in previous paper (Bruss *et al.* 1976). By means of isotope flux measurements, the steady-state clamping current for different voltages was split into its individual ionic components. The $I_{\text{Cl}}-V$ relation obtained by this analysis is reproduced in Fig. 7 D. By these different ways of estimating the $I_{\text{Cl}}-V$ curve of the cellular path-

TABLE II T_1 values (for definition of T_1 see text) of conductance increase after step voltage change from spontaneous potential to a prespecified clamping potential

Animal no.	Spontaneous potential mV	T_1 seconds				
		Clamping potential mV ...	-90	-80	-70	-50 -30
1	-19 to -26		39	42	42	60 102
2	-24 to -27 ^a		48	53	53	66 95
2	-24 to -27 ^a		50	54	53	70 90
3	-24 to -28 ^a		40	—	51	108 —
4	-22 to -25 ^a		60	—	53	120 —
5	-17 ^a		33	—	50	55 —
5	-2 ^c		28	—	60	120 —
6	-16 to -19 ^a		60	—	70	105 —
7	-27 to -28 ^a		40	—	80	90 —

^a NaCl-Ringer's outside.^b NaCl Ringer's outside + 60 μ M amiloride

KCl Ringer's outside.

different experimental conditions. Although the absolute values for equal voltage cond vary from skin to skin the general picture is quite uniform. T_1 ranges from about 30-120 s, the smallest values invariably are obtained for the largest command voltage steps.

Steady-state conductance values (g_{ss}^0) are reached between $t = 3$ min and $t = 5$ min, depending on voltage. In Fig. 6 the variation of steady-state conductance with clamping voltage under different experimental conditions is depicted. In the following these curves are denoted g_{ss}^0 -V curves. The g_{ss}^0 -V curve for skins exposed to NaCl Ringer's outside is S-shaped, with a maximum g_{ss}^0 at V well below the resting potential and the minimum above zero mV (Fig. 6 A and B). The main characteristics of the g_{ss}^0 -V curve is not affected by application of 60 μ M amiloride outside, or by substitution of outside sodium by potassium (Fig. 6 C and D). This gives strong support to the view that the sigmoidal g_{ss}^0 -V relation is not due to sodium channel processes, but is a characteristic feature of a chloride conducting pathway. The very different g_{ss}^0 -V curve obtained with a chloride free outer solution supports this hypothesis (Fig. 6 F).

Ouabain added to the solution bathing the inside of the frog skin reduces the active trans-epithelial sodium transport by inhibiting the active Na/K pump located at the inward facing membrane (Koefoed-Johnsen 1957, Dörge and Nagel 1977). Addition of ouabain to toad skin likewise inhibits, although slowly the active sodium transport. In the skin from which the results shown in Fig. 6 E are obtained the short circuit current was reduced from 18 μ A/cm² to 1 μ A/cm² after 3 h of exposure to 10⁻⁶ M ouabain. However, even after this treatment the S-shaped g_{ss}^0 -V curve was retained.

Relation between steady state chloride current and transepithelial potential difference

Steady state current values are depicted as a function of clamping voltage in Fig. 7 A, B and C. These steady-state I-V curves are non-linear and show outward going rectification as can be deduced from the g_{ss}^0 -V relations discussed above. Blocking the sodium channels with amiloride interferes selectively with the transcellular sodium current. Another way of elim-

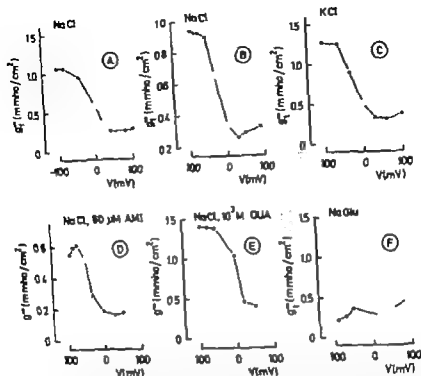


Fig. 6. Examples of steady-state total skin conductance (g_T^0) as a function of trans-epithelial clamping voltage (V). The outside Ringer's solutions are indicated for each curve. Results from the anion-free treated skin were obtained 30 sec after addition of the drug to the water solution. Ouabain was added to the lower bathing solution and the measurements performed after 3 h of drug exposure.

esting the sodium current is to replace sodium by potassium in the solution bathing the outside of the skin. Thus, the I - V curve obtained under either of these conditions comes close to that of the shunt pathway plus the cellular chloride conducting pathway. In general, the shunt pathway in toad skin exposed to chloride Ringer's on both sides has linear I - V characteristics for the voltage range investigated, $-80 \text{ mV} < V < +40 \text{ mV}$ (Bruus *et al.* 1976). Thus after inactivation of sodium channels we have

$$I_{\text{clamping}} = I_{\text{Cl}} + V g_{\text{shunt}} \quad (1)$$

here I_{clamping} is the steady-state clamping current at voltage V , g_{shunt} is the shunt conductance, and I_{Cl} is the steady-state chloride current through the voltage dependent cellular pathway. For each skin, g_{shunt} may be approximated by the skin conductance obtained with K-glucosate-Ringer's outside (cf. p. 3). Taking I_{clamping} values from curves B and C of Fig. 7 and using the g_{shunt} estimated as described above, we have calculated the steady-state current voltage curve of the chloride conducting pathway see Fig. 7 E and F. Another method of determining the I_{Cl} - V relation was suggested in a previous paper (Bruus *et al.* 1976). By means of isotope flux measurements, the steady-state clamping current for different voltages was split into its individual ionic components. The I_{Cl} - V relation obtained by this analysis is reproduced in Fig. 7 D. By these different ways of estimating the I_{Cl} - V curve of the cellular path-

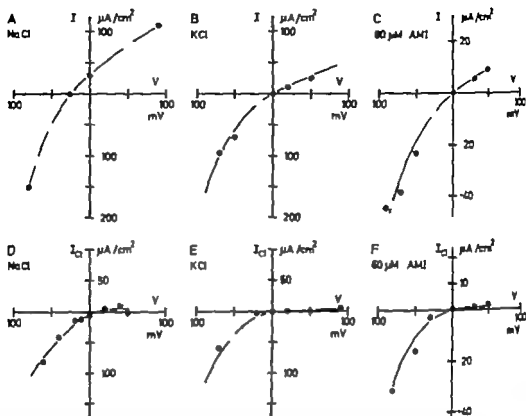


Fig. 7. *A, B* and *C*. Steady-state current-voltage relations of toad skin exposed to: *A*, NaCl Ringer's outside; *B*, KCl-Ringer's outside; and *C*, NaCl Ringer's outside with 80 μ M amiloride. *D, E* and *F*. Steady-state current-voltage relation of the cellular chloride conductive pathway. *D*, I_{Cl} calculated from isotope flux measurements, data from Bruus *et al.* 1976. *E* and *F*, I_{Cl} obtained from steady-state total currents corrected for the shunt component (*B, E*, $S_{shunt} = 0.50 \text{ mmho cm}^{-2}$; *C, F*, $S_{shunt} = 0.14 \text{ mmho cm}^{-2}$). For further explanation see text.

way we arrive at essentially the same result: namely that the cellular conductive chloride pathway exhibits a pronounced outward going rectification (in terms of current) with practically zero chloride current for $V \sim 0 \text{ mV}$. This means that the steady-state flux of chloride through this pathway becomes negligible as the transepithelial potential difference is decreased and reversed.

"Instantaneous" relation between current and transepithelial potential difference

In a series of experiments, the skin was continuously clamped on a preselected transepithelial potential difference, the holding potential. When a constant clamping current was attained, the voltage was changed in a staircase fashion, in 10–15 steps of 20 or 10 mV and of 50 ms duration, while the resulting current responses were recorded. Accordingly, the current-voltage curve obtained by this method is recorded within a time interval 1–2 orders of magnitude shorter than T_1 of the time-dependent conductance changes described above. We can therefore assume that the steady-state chloride conductance prevailing at the holding potential is not significantly influenced by the voltage staircase applied. Examples of such "instantaneous" I - V curves are shown in Fig. 8. The current now varies linearly with voltage. Note that the "instantaneous" I - V curve obtained from a holding potential of about -100 mV

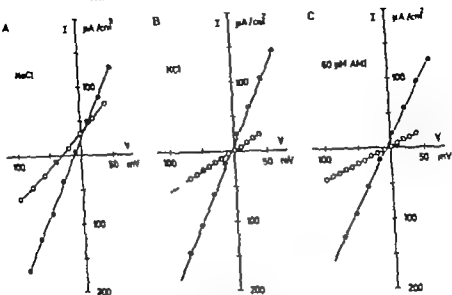


Fig. 8. Instantaneous current-voltage relations of toad skin. A. NaCl-Ringer outside, \bullet Holding potential, $V_h = 90$ mV \circ $V_h = 40$ mV. B. KCl-Ringer outside, \bullet $V_h = 90$ mV \circ $V_h = 40$ mV. C. NaCl-Ringer with $60 \mu\text{M}$ amiloride outside, \bullet $V_h = -97$ mV \circ $V_h = 40$ mV.

is linear even for small currents, and that this relationship prevails in sodium-free outside bathing solution and in skins exposed to $60 \mu\text{M}$ amiloride outside. Furthermore from Fig. 8 B and C it can be seen that only a minor fraction of this current goes through the shunt pathway as is apparent from the instantaneous I-V curves displayed from a holding potential of -40 mV in which situation the cellular chloride conductance is close to zero (Fig. 7 D E, and F). These results show therefore, that the activated conductive chloride pathway does not rectify and that the inward current through the activated pathway is carried by an outward flux of chloride ions across the membranes facing the outer bulk solution.

Discussion

In this and in an earlier paper (Bruns, Kristensen and Hvidt Larsen 1976) we have presented experimental evidence that the potential difference across toad skin affects the chloride conductance of a cellular pathway. Important features of this potential dependence have been recognized in the present study.

Upon a step-wise change of the transepithelial potential difference, the change in chloride conductance towards its new steady state proceeds slowly with a rise time of the order of minutes. The time dependent behaviour is not simply exponential, but exhibits an initial delay. The rise time of the conductance change depends on the magnitude of the voltage change, it is reduced as the step voltage change is increased. Furthermore, we have shown that the relation between steady-state chloride conductance and transepithelial potential difference is S-shaped. Highest conductance values are observed at potential differences more negative than the spontaneous potential whereas the chloride conductance approaches zero

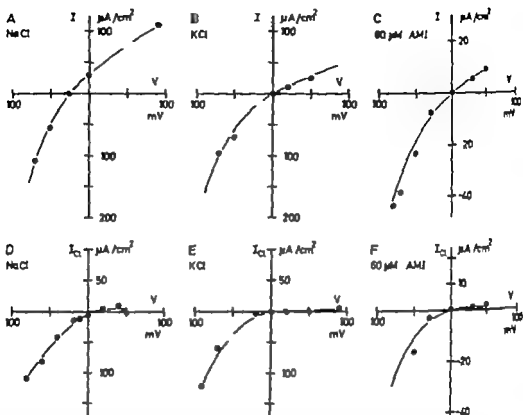


Fig. 7. *A*, *B* and *C* Steady-state current-voltage relations of load skin exposed to *A*, NaCl-Ringer's outside, *B* KCl-Ringer's outside and *C* NaCl-Ringer's outside with $60 \mu\text{M}$ miloride. *D*, *E* and *F* Steady-state current-voltage relation of the cellular chloride conducting pathway. *D*, I_{Cl} calculated from isotope flux measurements, data from Broux *et al.* 1976. *E* and *F*, I_{Cl} obtained from steady-state total currents corrected for the shunt component (*B*, *E*, $S_{\text{shunt}} = 0.50 \text{ mmho cm}^{-2}$; *C*, *F*, $S_{\text{shunt}} = 0.14 \text{ mmho cm}^{-2}$). For further explanation see text.

way we arrive at essentially the same result, namely that the cellular conductive chloride pathway exhibits a pronounced outward going rectification (in terms of current) with practically zero chloride current for $V > 0$ mV. This means that the steady-state flux of chloride through this pathway becomes negligible as the transepithelial potential difference is decreased and reversed.

"Instantaneous" relation between current and transepithelial potential difference

In a series of experiments, the skin was continuously clamped on a preselected transepithelial potential difference, the holding potential. When a constant clamping current was attained, the voltage was changed in a staircase fashion, in 10–15 steps of 20 or 10 mV and of 50 mV duration, while the resulting current responses were recorded. Accordingly the current-voltage curve obtained by this method is recorded within a time interval 1–2 orders of magnitude shorter than T_1 of the time-dependent conductance changes described above. We can therefore assume that the steady-state chloride conductance prevailing at the holding potential is not significantly influenced by the voltage staircase applied. Examples of such "instantaneous" I - V curves are shown in Fig. 8. The current now varies linearly with voltage. Note that the "instantaneous" I - V curve obtained from a holding potential of about

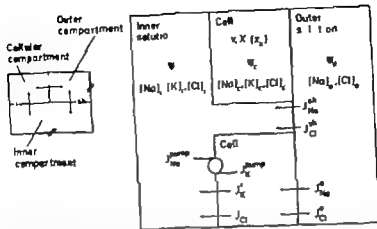


Fig. 9. Left: Diagrammatical representation of the three-compartment model. The compartments are separated by the physical barriers denoted, σ , outward facing membrane, σ , inward facing membrane, and σ , lateral membrane. The inner and outer compartments are assumed infinitely large. Right: Outline of the mathematical representation of the model. V , cell volume; $X_i (X_o)$, amount of intracellular, non-diffusible molecules; \bar{v}_i the mean valency; ψ_i , electrical potentials; J , fluxes. Concentrations are indicated by brackets.

Here, $[Cl]_o$ and $[Cl]_i$ are the outer and cellular chloride concentrations, V , the outer membrane potential ($\psi_o - \psi_i$), m_{Cl} the molar mobility of chloride within the membrane, β the partition coefficient, and δ the membrane thickness. F , R , and T have their usual significance. A similar equation may be assumed to describe J_K . It is now widely recognized that a non-linear steady-state $I_{Cl}-V$ curve may be due to voltage dependence of $[Cl]_i$ in combination with the fact that the membrane potentials vary. Another, and less trivial possibility would be that P_{Cl} changes in response to changes in membrane potential ("gating"). As an approach to estimating the significance of the "trivial" possibilities, we shall calculate theoretical current-voltage curves of an epithelial cell layer provided with properties of the toad skin.

A three-compartment model

The quantitative analyses will be based upon the three-compartment model shown in Fig. 9. This representation of the transporting cells is in accordance with the two-membrane hypothesis (Kosfeld-Johansen and Ussing 1958; Ussing and Windhager 1964) supplied with number of simplifying assumptions:

- Fluxes of only three ionic species are dealt with, sodium, potassium and chloride. Since all other ions are present at relatively low concentrations their contributions to the membrane currents are ignored.
- Intracellularly, number of larger molecules (X_i) are present which cannot pass the surface membrane of the cell. Their mean valency \bar{v}_i .
- The K^+ -permeability of the outer and facing membrane and the Na^+ -permeability of the inward facing membrane are assumed zero. Electrophysiological studies (Kosfeld-Johansen and Ussing 1958; Lindley and Hoshida 1964; Fachi *et al.* 1977) as well as studies of the cell volume response to osmotic variations and Na^+ - K^+ substitutions of the bathing solutions (MacRobbie and Ussing 1961) show that the K^+ -movement through the outer membrane and the passive Na^+ -movement across the inner membrane is at least one order of magnitude slower than that of other penetrating ions. Furthermore, the transepithelial potassium flux and the passive component of the transepithelial sodium flux seem to follow an extra-cellular route (Mandel and Curren 1972; Brown *et al.* 1976). As a consequence of these findings, we have decided to ignore completely the passive sodium current across the inner membrane and the potassium current across the outer membrane.
- All of the transcellular chloride current is assumed to pass through the sodium transporting cells. One type of evidence has been discussed on p. 12 in this paper. Further evidence will be given elsewhere (Krissemann 1977).

The strategy of the analysis is now to find proper equations for the individual membrane currents including the kinetics of the active Na^+/K^+ -pump, subsequently to specify mathematically the steady-state currents, and finally to solve the equations for selected values of the independent variables. The equations

as the potential difference is decreased and reversed. This gives rise to a steady-state I_{Cl} - V characteristics exhibiting a pronounced rectification with almost zero steady-state net flux of chloride in outward direction. The rectifying property however is a steady-state phenomenon. In a situation where the transcellular chloride conductance is maximum, the instantaneous cellular current varies linearly with the transepithelial potential difference, and the I - V curve proceeds with the same slope for currents carried by an inward and outward flux of chloride ions across the outer limiting membranes of the transporting cells.

The cellular chloride conducting pathway is insensitive to amiloride and shows the same voltage dependent behaviour when the sodium channels are blocked by amiloride. Therefore, the cellular chloride conducting pathway operates in parallel to the amiloride blockable sodium channels of the outward facing membrane.

By comparing the time courses of chloride and sodium currents to a sudden change in the outside concentration, the position of the cells through which the chloride current passes can be determined relative to the sodium transporting cells. The chloride current response to removal of outside chloride is exemplified in Figs. 2 B and 4 A, whereas the sodium current response to reduction of outside sodium concentration to zero mM is shown in Fig. 4 B. It can be seen that the decrease in chloride current is as fast as the decrease in sodium current, although the half times of these current responses vary from skin to skin. The reason for these rather slow and varying current responses is obscure. It is unlikely that they are due to the presence of a simple unstirred layer on top of the skin (see methods). Without overrating the resolution of this type of experiment, the results show however that the chloride pathway is located to cells within a distance from the outer bulk solution comparable to that of the sodium transporting cells. In other words they cannot be close to the subepidermal gland cells which are assumed to contribute to the adrenaline stimulated (outward) chloride transport (Koefoed-Johnsen *et al.* 1952).

The variables which may affect the transcellular chloride conductance

In the experimental approach presented here, the ionic composition of the solutions bathing the inner and the outer surface of the skin—as well as the transepithelial potential difference—are fully controlled by us. Cellular compartments within the skin, however may change ionic composition and volume during voltage clamping. Therefore, in considering the nature of the voltage dependence of the transcellular chloride conductance, we have to take into account that variables other than the transepithelial potential difference may change when the skins are submitted to voltage clamping.

A transcellular chloride current is governed by two conductances in series, g_1 the conductance of the outward facing membrane and g_2 that of the inward facing membrane. Thus, the transcellular chloride conductance (g_{Cl}) is given by

$$\frac{1}{g_{Cl}} = \frac{1}{g_1} + \frac{1}{g_2} \quad (2)$$

The individual membrane conductances are functions of ion concentrations of the bathing solutions, membrane potential, and the mobility of the ion within the membrane. Even for the most simple case—the homogenous membrane with a linear electrical potential profile—this dependence comes out as a rather complex equation (see *for example* Finkelstein and Mauro 1963). Taking g_{Cl} as an example and considering the sign convention used in the present paper this dependence reads

$$g_{Cl} = \frac{P_{Cl}^0 F^2 V}{R T} \frac{[Cl]_o - [Cl]_i \epsilon_0}{(1 - \epsilon_0) \ln([Cl]_o/[Cl]_i \epsilon_0)} \quad (3)$$

where $\epsilon_0 = \exp(-FV/RT)$, and $P_{Cl}^0 = \alpha_{Cl} RT/\beta d$

TABLE III. Computed dependent variables of interest for the discussion for 3 values of the transepithelial clamping voltage. The independent variables used in these computations are listed in the legend to Fig. 10. The open circuit potential, $V_{\text{open}} = -33.5$ mV

V (mV)	-100	0	+50
I_{steady} ($\mu\text{A cm}^{-2}$)	-124.1	35.2	77.4
I_{Cl} ($\mu\text{A cm}^{-2}$)	-125.6	0	22.8
$\frac{I_{\text{Cl}}}{I_{\text{total}}}$ (ratio cm^{-2})	2.00	1.00	0.92
V_p (mV)	2.8	78.4	115.3
$[\text{Cl}]_o$ (mM)	15.9	5.4	5.4
J ($\text{mmol cm}^{-2} \text{ sec}^{-1}$)	1.6	9.0	17.9

changes is expected to increase the chloride conductance of the outward facing membrane. Our calculations show that these changes taken together result in an increase of the transepithelial chloride conductance by a factor of 2.8, despite that P_{Cl}^i and P_{Cl}^o are kept constant. By itself this is not a truly result, because it illustrates that a strong asymmetry may be inherent in a cellular chloride pathway even if this pathway is assumed to be of the most simple nature, and the skin is exposed to Ringer's of identical composition on its two sides.

From Fig. 10 B, however, it can be seen that the S-shaped $g_{\text{Cl}}^{\text{eff}}$ V relation typically observed (Fig. 6) cannot be reproduced by the model. Another significant deviation from the observed data is that the very large negative chloride currents for $V < -70$ mV presuppose such large chloride permeabilities that also the chloride current for $V > 0$ mV amounts to a relatively large fraction of the total current. In the example shown in Fig. 10 A, $I_{\text{Cl}} = 23 \mu\text{A/cm}^2$ at $V = 50$ mV. In contrast, our measurements show that the steady-state chloride current in a toad skin clamped to this voltage is not significantly different from zero. In other words, the observed strong outward going rectification of the cellular chloride pathway cannot be obtained from this simple model. It is likely therefore, that a chloride permeability varies reversibly with the transepithelial potential difference, i.e. it increases at hyperpolarizing clamping voltages and decreases following depolarization and reversal of the transepithelial potential difference. This possibility will be considered further in the following section.

Evidence of chloride permeability which varies reversibly with the transepithelial potential difference

In the computed example discussed above, the unidirectional chloride fluxes come out to be about $9 \text{ mmol cm}^{-2} \text{ min}^{-1}$ under short circuit conditions. Chloride fluxes of this magnitude are within the range of fluxes observed in the toad skin clamped to zero mV. These observed chloride fluxes, however, do not seem to be of a simple passive nature as is assumed in the computations. The first point of interest here is that both the chloride influx and outflux is practically uninfluenced by the transepithelial potential difference in the range $0 \text{ mV} < V < 50$ mV (Braun *et al.*, 1976, Fig. 6). This observation together with the dependence of the chloride outflux on the chloride concentration of the outer solution led us to suggest that the larger fraction of the chloride outflux passes the skin by way of a Cl^-/Cl^- exchange. In Table IV is collected steady-state fluxes measured in skins from 10 different toads clamped at $V = 50$ mV.

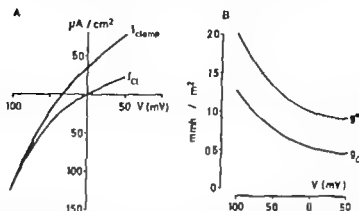


Fig. 10 A Computed voltage dependence of the steady-state transepithelial current (I_{tump}) and the steady-state transcellular chloride current (I_{Cl}). B Computed g_{Cl}^o -V and g_{Cl}^i -V relations. For these calculations the following independent variables were used. $[Na]_o = [Na]_i = 115$ mM $[Cl]_o = [Cl]_i = 117$ mM $[K]_i = 2$ mM Permeability coefficients and pump constants in $(10^{-4} \text{ cm}^2 \text{ s}^{-1})$ $P^o = 1$ $P_{Cl}^o = 12$ $P_K^o = 50$ $P_{Na}^o = 25$ $P_{Cl}^i = 0.08$ $A_K = 9$ $A_{Na} = 6$ $X_o = 30$ nanomole $z_x = -1$ eqv/mole $\sigma_{HCl} = 0.95$ $T = 295$ K.

tions and the computer analysis are treated in the Appendix to this paper where also the validity of the assumptions underlying the various equations is discussed. Here we shall proceed with the representation and discussion of the results of the analysis.

Theoretical steady-state I-V curves obtained under the assumption of constant permeability

During the initial examination of the transport model we tried several combinations of permeability coefficients and analysed for the shape and position of the steady-state I-V curve. It soon became clear that only for combinations of permeability coefficients where $P_{Cl} > P_i$ and $P_{Cl}^i < P_K^i$ the I-V curve is non linear with a large I_{Cl} component at hyperpolarizing voltages. With this restriction, the absolute values of the permeability coefficients were selected in order to obtain approximately (1) the experimentally determined short circuit current, (2) the measured open circuit potential, and (3) a ratio of $R_o/R_i = 0.8$, where R_o and R_i are the outer membrane and the transcellular resistance, respectively. This last mentioned assumption agrees with results of micro-electrode studies of frog skin exposed to NaCl Ringer's on both sides (Ussing and Windhager 1964; Lindemann and Thomsen 1967).

Examples of computed I-V curves are shown in Fig. 10. The legend to Fig. 10 contains the independent variables on which these calculations are based. The dependent variables of interest for our discussion are given for three selected clamping voltages in Table III.

Comparison of Fig. 10 with Fig. 7 reveals that the model reproduces I-V curves displaying outward going rectification with a large I_{Cl} component at hyperpolarizing clamping voltage which resemble those obtained in the *in vitro* preparation. From the values given in Table II it can be seen that in the voltage range 50 mV to -100 mV the chloride conductance increase is due to (1) a decrease and a reversal of the outer membrane potential, and (2) a relatively large increase in $[Cl]_o$. By comparing with eqn. (3) it is seen that each of these

House (1971) investigated the response of the transepithelial potential difference to small changes in ion concentrations of the outer solution, and he concluded that the chloride permeability of the outside border is larger than its sodium permeability. However the assumption that $P^o < P_K^o$ may not be realistic in the frog skin epithelium where the distribution across the inner membranes is far from thermodynamic equilibrium (MacRobbie & Ussing 1961). Calculations show that this requires $P^o > P_K^o$.

during the non-steady-state period following a step voltage change. By itself this increase in $[Cl]_i$ is expected to increase the conductance of the cellular chloride pathway (cf. equ. (3)). It cannot be assumed, therefore, that the conductance changes shown in Fig. 5 are caused by permeability changes only. This complication also makes it difficult to identify the variable which controls the chloride permeability. The permeability activation may be initiated by a change in membrane potential, or by a change in the concentration of an intracellular ion in which case the ion must be assumed to participate in a permeability controlling reaction step. From the type of experiments presented in this paper we cannot distinguish between these possibilities.

Appendix

The variables of the model presented in Fig. 9 of the discussion section are divided into independent and dependent variables, as the listing in Table I A and Table II A. The interrelationship between these variables will be explained below.

Equations of passive fluxes

Throughout we will assume that the passive fluxes of Na⁺, K⁺ and Cl⁻ are given by the Nernst-Planck equation integrated under the assumption of constant electrical field within the individual surface membranes (Goldstein 1943; Hodgkin and Katz 1946). With the voltage and current sign notation used in the present paper the integrated flux equations read:

(1) Passive fluxes through the paracellular shunt pathway

$$J_j^p = \frac{P_j^p(x_j FV/RTD)}{1 - \exp(x_j FV/RTD)} ([j]_o - [j]_i \exp(x_j FV/RTD)) \quad (1A)$$

here $V = \psi - \psi_o$ and j is substituted by either Na⁺ or Cl⁻.

(2) Passive fluxes through the outward facing membrane

$$J^o = \frac{P_j^o(x_j FV_o/RTD)}{1 - \exp(x_j FV_o/RTD)} ([j]_o - [j]_i \exp(x_j FV_o/RTD)) \quad (2A)$$

here $V = \psi - \psi_o$ and j is either Na⁺ or Cl⁻.

(3) Passive fluxes through the inward facing membrane

$$J_i^p = \frac{P_j^i(x_j F(V - V_o)/RTD)}{1 - \exp(x_j F(V - V_o)/RTD)} ([j]_i - [j]_o \exp(x_j F(V - V_o)/RTD)) \quad (3A)$$

Here j either K⁺ or Cl⁻.

For small variations of V the paracellular fluxes in frog skin and toad skin are governed by eqn. (1A) (Mandel and Curtis 1972; Brown *et al.* 1974). For large positive values of V (reversal of the transapical potential difference) P_j^p and P_j^i are not constant. In the voltage range of interest for the present analysis, however, eqn. (1A) is a fairly good approximation. Also, it has been experimentally verified that the Na⁺ transport across the outer membrane in frog skin takes place according to eqn. (2A). The use of this equation, however, requires that $[Na]_o$ is kept constant, which is fulfilled here. If $[Na]_o$ varies P_{Na}^o must be replaced by $P_{Na, max}^o K_m / (K_m + [Na]_o)$, here $P_{Na, max}^o$ the Na⁺-permeability corresponding to $[Na]_o = 0$ M and K_m an empirical constant (Ficker *et al.* 1977). A dependence of P_{Na}^o on $[Na]_o$ (Baber, 1971; Hvidt-Larsen 1973; Ely and Smith, 1973; Movel and Leblanc, 1975) is not taken into account here. The application of the formalism above to K^+ , H^+ and H^+ has not yet been experimentally investigated. The use of eqns. (1A) and (3A), however, is not invalidated by our hypothesis that the larger fraction of the transcellular Cl⁻ flux goes on as exchange mechanism. For the calculation of current-voltage curves, intracellular ion concentrations, and cell volume, Cl⁻ chloride exchange will not appear in the equations, since these

TABLE IV Steady-state chloride influx ($J_{\text{Cl}}^{\text{in}}$) and outflux ($J_{\text{Cl}}^{\text{out}}$) through the isolated toad skin exposed to NaCl-Ringer's on both sides and clamped at $V = 50$ mV. In each experiment influx and outflux were obtained from laterally symmetrical skin halves from one toad. Data taken from Brøn *et al.* 1976.

Animal no	$J_{\text{Cl}}^{\text{in}}$ nmol cm ⁻² min	$J_{\text{Cl}}^{\text{out}}$	$J_{\text{Cl}}^{\text{in}}/J_{\text{Cl}}^{\text{out}}$	I_{Cl} μA cm ⁻²
1	4.0	3.60	1.1	-0.68
2	4.60	5.57	0.83	1.56
3	4.1	8.44	0.50	6.80
4	5.57	4.93	1.13	-1.03
5	2.57	3.28	0.78	1.14
6	2.73	2.96	0.9	0.37
7	4.44	5.27	0.84	1.33
8	3.93	3.56	0.73	-0.60
9	3.99	5.38	0.69	1.88
10	3.35	3.63	0.92	0.43
Mean			0.85	1.2
± S.E.			0.06	0.73

If the chloride fluxes are of a simple passive nature the flux ratio is given by (Ussing, 1949)

$$\frac{J_{\text{Cl}}^{\text{in}}}{J_{\text{Cl}}^{\text{out}}} = \frac{(\text{Cl})_o}{(\text{Cl})_i} \exp\left(\frac{-FV}{RT}\right)$$

where $(\text{Cl})_o$ and $(\text{Cl})_i$ are the chloride activities in the outer and inner bathing solutions. Under the prevailing experimental conditions the flux ratio is predicted to be 0.14. The experimentally determined flux ratio, however, amounts to 0.85 ± 0.06 , which is significantly different from 0.14 ($P < 0.05\%$). On the contrary the experimentally determined flux ratio is only slightly smaller than one, which is expected for a cellular Cl^-/Cl^- exchange in parallel to a low conductance extracellular route. Thus, the result of this flux ratio analysis is consistent with the hypothesis that an exchange mechanism governs the transcellular chloride transport for $V_{\text{clamp}} > 0$ mV. In other words, the steady-state permeability of the conductive cellular pathway must be close to zero in this voltage range.

As revealed by the "instantaneous" $I-V$ curves shown in Fig. 8 the conductive chloride pathway allows a large inward chloride current (outward chloride flux) to pass if the skin has been hyperpolarized for a few minutes. We therefore conclude that the steady-state permeability of the conductive chloride pathway is a function of the transepithelial potential difference.

The time course of conductance change

The kinetics of the permeability activation are probably reflected in the time course of total conductance increase following hyperpolarization of the skin (Fig. 5). Thus, the activation reaction seems to be a slow process, proceeding with a time constant of no less than 20 s. This makes it very likely that the concentration of chloride in the cellular compartment increases

Note that the voltage sign notation in the paper quoted is opposite to the one used in the present paper.

Steady-state criteria

These concern the requirements of constant (time-independent) concentration of any of the intracellular species, electroneutrality and osmotic equilibrium between the intracellular and the outer compartment. Mathematically these criteria are expressed by the following equations.

(1) *Constant intracellular concentrations*

$$\sum j_i - \sum J_i = 0, \quad (6A)$$

here the summation is taken over the individual pathways, i is the total flux of the ion i across the outer membrane is equal to the total flux of this ion across the inner membrane.

(2) *Electroneutrality*

$$[Na]_i + [K]_i + z_2 X_2 / v - [Cl]_i = 0 \quad (7A)$$

(3) *Osmotic equilibrium*

The epithelial cell layer of frog skin *in situ* if the toxicity of the solution bathing the inner border is lowered. In contrast, the epithelial cell volume (the SO_4 Ringer's) is independent on the toxicity of the outer solution (MacRobbie and Ussing 1961). Accordingly we shall assume that the cells in steady-state are osmotically equilibrated with the outer solution. The fact that both P_K and P_{Cl} is different from zero implies that KCl probably exhibits reflection coefficient (σ_{KCl}) which is different from unity. Taking this into account, the requirement of osmotic equilibrium between the cellular and the inner compartment leads to the following equation

$$([K]_i - [Na]_i - (2\sigma_{KCl} - 1)[Cl]_i + X_2/v) = ([Na]_e + (2\sigma_{KCl} - 1)[K]_e - [Cl]_e) \quad (8A)$$

Combination of eqns. (7A) and (8A) gives the expression of the steady-state volume of the transporting cell

$$v = \frac{(1 - z_2) X_2}{2(\sigma_{KCl} - 1)([K]_e + 2[Cl]_e - \sigma_{KCl}[Cl]_e)} \quad (9A)$$

The inner membrane reflection coefficient for KCl has not been experimentally determined. In the example presented in the discussion section we arbitrarily assumed $\sigma_{KCl} = 0.95$. Choosing $\sigma_{KCl} = 0.5$ however has only slight effect on the dependent variables of interest here. Thus, the conclusions drawn in the discussion section are not influenced by our choice of σ_{KCl} .

Procedure of numerical solution

Proper combination of the above equations leads to an equation of the form,

$$F(V_0) = 0, \quad (10A)$$

which transcendental in V and, therefore, cannot be solved analytically. For its numerical solution we used Newton method of successive approximations, here the difference between new guess (V^{n+1}) to eqn. (10A) and the immediately preceding one (V^n) is given by

$$V^{n+1} = V^n - (F(V_0)/F'(V_0)) \quad V^{n+1}$$

here $F'(V_0)$ the first derivative of $F(V_0)$. The iteration was stopped when

$$|V^{n+1} - V^n| \leq 2.5 \cdot 10^{-8} \text{ mV}$$

A closer examination of the function $F(V_0)$ revealed that it has only one solution for each set of independent variables.

After the correct value of V found the other dependent variables listed in Table II A are calculated for this V . It is easily recognized, however that large number of other steady-state parameters can be calculated too. The steady-state clamping current (I_{clamp}) and the steady-state cellular chloride current are given by

$$I_{clamp} = z_{Na} F(J_{Na} + J_{Na}^0) - z_{Cl} F(J_{Cl} - J_{Cl}^0) \quad (11A)$$

$$I_{Cl} = z_{Cl} F J_{Cl} \quad (12A)$$

which of course are of particular interest for the present study

TABLE I A. Listing of the independent variables of the model.

Symbol	Definition
$[Na]_o, [Cl]_o$	Concentration of Na ⁺ and Cl ⁻ in outer compartment
$[Na]_i, [Cl]_i, [K]_i$	Concentration of Na ⁺ , Cl ⁻ and K ⁺ in inner compartment
P_{Na}^s, P_{Cl}^s	Na ⁺ and Cl ⁻ permeability coefficient of shunt membrane
P_{Na}^i, P_{Cl}^i	Na ⁺ and Cl ⁻ permeability coefficient of outer membrane
P_K, P_{Cl}^i	K ⁺ and Cl ⁻ permeability coefficient of inner membrane
A_{Na}, A_K	Pump coefficients defined by eqns. (4 A) and (5 A)
X_o	Amount of intracellular non-diffusible molecules
z_x	Their mean valency
σ_{KCl}	KCl reflection coefficient of inner membrane
V	Transmembrane potential difference
T	Temperature in K

Throughout the standard symbols for the Faraday (F), gas constant (R) and valency of the ion species (z_i) are used

Active fluxes through the in- and facing membrane

The general form of the pump flux equations will be

$$J_{Na}^{pump} = A_{Na} [Na]_o \quad (4 A)$$

$$J_{K}^{pump} = -A_K [Na]_o \quad (5 A)$$

where A_{Na} and A_K are positive constants. As is seen, the active flux of Na⁺ is assumed proportional to the intracellular Na⁺ concentration. This is a good approximation in the voltage range $V < 0$ mV, here $[Na]_i$ is small. In cases where P_{Na}^s is high and $V > 0$ mV the pump saturates. Saturation, however, is not expected to occur within the range of $[Na]_o$ variation of interest for the present analysis. (A model analysis based upon saturation kinetics of the Na/K pump is discussed in Hvid Larsen 1978.) Furthermore, we assume a fixed coupling ratio of 3:2 between the active Na⁺ flux and the active K⁺ flux (Thomas 1972). Evidence for varying Na/K coupling ratio has been obtained by Brinley and Mullins (1974) working with the squid axon. Using other ratios of pump fluxes taken from their study, however, does not affect the conclusions emphasized in the discussion. Finally, we assume that the active transport mechanism operates independently of the inner membrane potential. This assumption is valid for the squid axon for variation of V between -10 and -90 mV (Brinley and Mullins 1974). It should be added that due to the high resistance of the outer membrane as compared to that of the inner one, the variation of the inner membrane potential is expected to be small and about one order of magnitude less than the voltage changes imposed on the shunt.

TABLE II A. Listing of the dependent variables used in this paper

Symbol	Definition
$[Na]_o, [Cl]_o, [K]_o$	Concentration of Na ⁺ , Cl ⁻ and K ⁺ in the cellular compartment
$J_{Na}^s, J_{Cl}^s, J_K^s$	Passive fluxes of Na ⁺ through the shunt (sh), outer (o), and inner (i) membrane
$J_{Na}^i, J_{Cl}^i, J_K^i$	Membrane currents of Na ⁺ through the membranes sh, o, and i
I_{total}	Total transepithelial current
G_{sh}, G_o, G_i	Integral conductance of the ionic pathways of the membranes sh, o, and i
G_{total}	Total transepithelial conductance
G_{Cl}^i	Transcellular chloride conductance
$E_{Na}^s, E_{Cl}^s, E_K^s$	Equilibrium potential of Na ⁺ across the membranes sh, o, and i
J_{Na}^{pump}	Active flux of Na ⁺ through the inner membrane
J_K^{pump}	Active flux of K ⁺ through the inner membrane
V	Outer membrane potential ($-V = V_o - V_i$)
ω	Cell water volume
J_{Cl}^{total}	Transepithelial and rectal outflux of Cl ⁻
J_{Cl}^i	Transepithelial unidirectional flux of Cl ⁻

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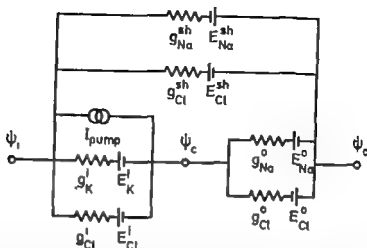


Fig. A 1. Electrical circuit analogue of the three-compartment model. The upper indices to the conductances and electromotive forces indicate the outward facing membrane, the inward facing membrane, and the shunt membrane. The rheogenic Na/K pump located in the inward facing membrane is equivalized to a current source. The pump current (I_{pump}) is dependent on the cellular Na⁺-concentration, but independent of the inner membrane potential.

The electrical circuit analogue and conductance equations

The choice of electrical circuit representing the three-compartment model depends on the structure of the membranes (Finkelstein and Mauro 1963). We will assume that each of the limiting membranes is mosaic, which means that it consists of regions exclusively permeable to Cl and other regions exclusively permeable to Na. It follows from this assumption that each of the ionic current components can be ascribed its own driving force and conductance. Accordingly the mixed equivalent circuit shown in Fig. A 1 will be used. It follows from the depicted geometrical arrangement of conductances that the steady-state transmembrane conductance ($g_{\text{Cl}}^{\text{ss}}$) is given by

$$g_{\text{Cl}}^{\text{ss}} = g^{\text{sh}} + g_{\text{Cl}}^{\text{sh}} + \frac{(g^{\text{o}} + g_{\text{Cl}}^{\text{o}})(g^{\text{i}} + g_{\text{Cl}}^{\text{i}})}{g_{\text{Na}}^{\text{o}} + g_{\text{Cl}}^{\text{o}} + g^{\text{i}} + g_{\text{Cl}}^{\text{i}}} \quad (13A)$$

For each choice of independent variables and subsequent determination of dependent variables, the value of the individual ion conductances (the integral conductance, g_j) is calculated from

$$g_j = I_j / (V_m - E_j), \quad V_m \neq E_j \quad (14A)$$

where $I_j (= z_j F J_j)$ is the membrane current carried by j , E_j the equilibrium potential of j calculated from the Nernst-equation, and V_m the membrane potential.

For the sake of completeness, we shall recall that for an ionic pathway with constant field properties, and provided $E_j \neq 0$, the instantaneous I_j - V curve is non-linear which means that the integral conductance, g_j , is voltage dependent. For the case of $g_{\text{Cl}}^{\text{ss}}$, this dependence is expressed mathematically in eqn. (3) on p. 13. Note that eqn. (14A) is not defined for $V_m = E_j$. With the constant field assumption and continuing with the $g_{\text{Cl}}^{\text{ss}}$ as the example, it can be shown that

$$g_{\text{Cl}}^{\text{ss}} = \frac{F^2}{R} \frac{V_m - E_{\text{Cl}}^{\text{ss}}}{T} \frac{[Cl]_o}{([Cl]_o - [Cl]_i)} \quad V_m \neq E_{\text{Cl}}^{\text{ss}} \quad (15A)$$

Our thanks are due to Mr Ole Bengtson for constructing and to Mr Benny Knutström for servicing the voltage clamp circuits, and to Dr Bjørn Rasmussen for his help during the development of the computer program. The fast voltage clamp circuit provided with staircase function was constructed and built for us by Abteilung für Membranforschung an Epithelen, Physiologie (II) der Universität des Saarlandes, Homburg/Saar BRD. It is a pleasure to thank professor Bernd Lindemann for allowing us to benefit from the expertise of his laboratory. Technical assistance by Mrs Lone Pedersen and Mrs Lisbet Sortkjær is appreciated. Purchase of electronic equipment was supported by a grant from the Danish Natural Science Research Council.

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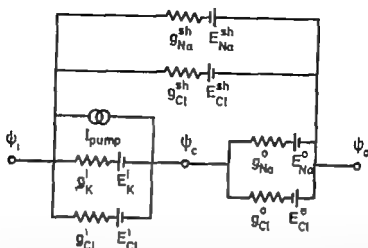


Fig. A 1. Electrical circuit analogue of the three-compartment model. The upper indices (*sh*) indicate the conductances and electromotive forces indicate, *o*, outward facing membrane, *i*, inward facing membrane and *sh*, shunt membrane. The rheogenic Na/K pump located in the inward facing membrane is equivalentized to a current source. The pump current (I_{pump}) is dependent on the cellular Na⁺-concentration, but independent of the inner membrane potential.

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$$g_{\text{Cl}}^{\text{CO}} = g_{\text{Na}}^{\text{sh}} + g_{\text{Cl}}^{\text{sh}} + \frac{(g_{\text{Na}}^{\text{sh}} + g_{\text{Cl}}^{\text{sh}})(g_{\text{K}}^{\text{i}} + g_{\text{Cl}}^{\text{i}})}{g_{\text{Na}}^{\text{sh}} + g_{\text{Cl}}^{\text{sh}} + g_{\text{K}}^{\text{i}} + g_{\text{Cl}}^{\text{i}}} \quad (13A)$$

For each choice of independent variables and subsequent determination of dependent variables, the value of the individual ion conductances (the integral conductance, g_j) is calculated from

$$g_j = I_j / (V_m - E_j), \quad V_m = E_j \quad (14A)$$

where $I_j (= z_j F J_j)$ is the membrane current carried by j , E_j the equilibrium potential of j calculated from the Nernst-equation, and V_m the membrane potential.

For the sake of completeness, we shall recall that for an ionic pathway with constant field properties, and provided $E_j \neq 0$, the instantaneous I_j - V curve is non-linear which means that the integral conductance, g_j , is voltage dependent. For the case of g_{Cl} this dependence is expressed mathematically in eqn. (3) on p. 13. Note that eqn. (14A) is not defined for $V_m = E_j$. With the constant field assumption and continuing with g_{Cl} as the example, it can be shown that

$$g_{\text{Cl}} = \frac{F^2 C_{\text{Cl}}^{\text{i}} V_0 [\text{Cl}]_{\text{o}} [\text{Cl}]_{\text{i}}}{R T ([\text{Cl}]_{\text{o}} - [\text{Cl}]_{\text{i}})} V - E_{\text{Cl}}^{\text{o}} [\text{Cl}]_{\text{o}} + [\text{Cl}]_{\text{i}} \quad (15A)$$

Our thanks are due to Mr Ole Bengtson for constructing and to Mr Benny Knudström for servicing the voltage clamp circuits, and to Dr Bjørn Rasmussen for his help during the development of the computer program. The fast voltage clamp circuit provided with staircase function was constructed and built for us by Abteilung für Membranforschung an Epithelien, Physiologie (II) der Universität des Saarlandes, Homburg/Saar BRD. It is a pleasure to thank professor Bernd Lindemann for allowing us to benefit from the expertise of his laboratory. Technical assistance by Mrs Lone Pedersen and Mrs Lisbet Sørkjær is appreciated. Purchase of electronic equipment was supported by a grant from the Danish National Science Research

It was the purpose of the experiments described below to obtain additional information on the possibility of a common molecular basis for the chloride exchange diffusion pathway and the pathway responsible for the conductive transfer of chloride under hyperpolarization. This was achieved by comparing the effects of some inhibitors and of ionic substitutions on chloride efflux under short circuit conditions with the effects of these inhibitors and ionic substitutions on clamping current during hyperpolarization.

Some of the experiments have already been reported recently (Kristensen and Hvild Larsen 1976).

Methods

The experimental details, including storage and treatment of the toads (*Bufo bufo*) and the chloride flux measurements were described in a previous communication (Bruus *et al.* 1976).

The chloride Ringer solution had the following composition: 113.5 mM Na, 1.9 mM K, 0.9 mM Ca^{++} , 14.8 mM Cl^- and 2.4 mM HCO_3^- . Bromide, iodide, nitrate, and gluconate Ringer solutions were prepared in the same way as the chloride Ringer solution.

Phloretin was obtained from K & K Laboratories (USA), and furosemide was kindly supplied by Hoechst Danmark A/S.

Transapical potential differences are given in this paper as $V = \varphi_{\text{in}} - \varphi_{\text{out}}$.

Results

In the experiments to be described we compare 2 modes of chloride transport across the isolated toad skin. A chloride-chloride exchange diffusion and a chloride transport with charge transfer. The exchange diffusion is studied under short circuit conditions, and can be measured quantitatively as the decrease in ^{36}Cl efflux observed, when the outside solution is changed from chloride to sulphate Ringer's. The conducting pathway is studied under conditions, where the transapical potential difference is clamped at values more negative than the spontaneous potential difference, which is about -20 mV in toad skins exposed to sodium chloride Ringer's outside. The cellular chloride current can then be estimated in two ways, either by measuring the influx of ^{36}Cl , or simply by recording the outward clamping current, since it was shown in the preceding paper (Hvild Larsen and Kristensen 1977) that for $V_{\text{clamp}} < -35$ mV the hyperpolarizing current is almost entirely carried by an outward transport of chloride.

The relations between the two types of chloride transport are studied by comparing their sensitivities to inhibitors and to substitution of chloride by other anions in the outer bathing medium.

Neither anion substitutions nor inhibitors can be assumed *a priori* to affect specifically the chloride pathways. In the present experiments, therefore, short circuit current was recorded in order to disclose effects on active sodium transport.

The efflux of chloride into an outside bathing solution of NaCl-Ringer's varies considerably from skin to skin, ranging in the present material from 1 to about 40 $\text{nmol cm}^{-2} \text{ min}^{-1}$. This efflux consists of two components, a cellular exchange diffusion component and a paracellular shunt component (Bruus *et al.* 1976). The shunt flux, however, is small and constant magnitude, ranging from 0.6 to 1.0 $\text{nmol cm}^{-2} \text{ min}^{-1}$ in the short circuited skin. A large reduction of chloride efflux caused by various treatments as presented below may

Relation between chloride exchange diffusion and a conductive chloride pathway across the isolated skin of the toad (*Bufo bufo*)

By

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Abstract

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Substitution of chloride in the outside bathing medium of the toad skin with bromide, iodide, nitrate and sulphate leads to a reduction in the apparent exchange diffusion of chloride across this tissue, and also to a reduction of the chloride current recorded during hyperpolarization. A series of inhibitors (thiocyanate, furosemide, phloretin, and acetazolamide) also affects chloride exchange diffusion, hyperpolarization current as well as chloride influx during hyperpolarization. Although in some cases, effects on short circuit current were also observed, none of the effects on chloride transport systems could be explained as secondary effects due to a primary interaction with the sodium transport mechanisms. A correlation was found between the clamping current recorded during hyperpolarization and the efflux of chloride under short circuit conditions with chloride Ringer on both sides. On the basis of these findings, and the results reported in the previous paper (Hvid Larsen and Kristensen 1977) it is considered probable that the membrane molecules responsible for chloride exchange diffusion under short circuit conditions, are rearranged under the influence of a hyperpolarizing clamping voltage, thereby forming channels allowing charge transferring transport of chloride.

The main fraction of transepithelial chloride transport across toad skin occurs via cellular pathways (Bruus *et al* 1976). In a previous paper the conductive pathway was characterized with respect to its voltage and time dependence. In skins clamped at a transepithelial voltage difference exceeding the spontaneous potential difference, the electrical properties of the epithelium were found to be governed by a cellular chloride conducting pathway (Hvid Larsen and Kristensen 1977). Under short circuit conditions, however the main fraction of chloride transport seems to occur via an electrically silent chloride exchange diffusion (Bruus *et al* 1976).

Since it was found (Bruus *et al* 1976) that acetazolamide partially inhibits transcellular conductance as well as chloride exchange diffusion, it is possible that common molecular structures are involved in both mechanisms of chloride transport.

TABLE II. The effect on clamping current of anion substitutions in the outside bathing solution. Inside solution is chloride Ringer's, inward current is considered positive.

Exp. no.	Clamping voltage mV	Clamping current ($\mu A \text{ cm}^{-2}$)		
		Cl ⁻ Ringer's	Br ⁻ Ringer	I ⁻ Ringer's
1	80	-44.3	-21.9	+2.5
	90	73.0	-30.0	+2.9
3	80	94.3	-78.6	+4.3
4	110	61.4	-55.7	-1.1
5	-110	18.6	-15.7	-11.4
6	110	-57.1	-39.3	-10.7
7	-110	371.4	-207.3	-32.9
8	-110	4.3	-19.3	-5.7

(Bruss *et al.* 1976), and the effluxes are equal to those measured with sulphate or gluconate Ringer's on the outside, *i.e.* they are estimates of the extracellular electro-diffusional transport of chloride, which was characterized in a previous communication (Bruss *et al.* 1976). Also substitution with bromide Ringer's leads to a significant reduction in chloride efflux, but in all skins, the remaining chloride efflux is larger with bromide on the outside than with nitrate. From this observation we conclude that bromide can exchange with chloride across the toad skin, but that it is not as good an exchange partner as chloride itself. The effluxes measured with iodide Ringer's on the outside cannot be distinguished from those measured with nitrate Ringer's, indicating that iodide does not exchange significantly with chloride. With respect to their ability to exchange with chloride, the anions tested can therefore be listed in the following order: Cl⁻ Br⁻ [≅ NO₃⁻].

Under short circuit conditions, anion substitutions not only affect chloride efflux, but moreover the short circuit current (I_{sc}) which is significantly increased, see Fig. 1. With respect to their effect on I_{sc} , the anions rank in the reverse order of their exchange capability. This observation was made in all 8 expts. of Table I. The spontaneous transepithelial potential difference measured with different anions in the outside solutions was: Chloride Ringer's, 24.3 mV; bromide Ringer's, -39.4 mV; iodide Ringer's, -72.0 mV; and nitrate Ringer's, 77.0 mV (averages of measurements in expts. of Table I).

The clamping currents measured during hyperpolarization in the above expts. are given in Table II. Clearly the largest currents are observed, when the outside is bathed with chloride Ringer's. The currents measured with iodide are very much smaller. Bromide holds an intermediate position, giving about 2/3 of the current observed with chloride in the outside bathing medium. Fig. 2 shows that amiloride, added to the outer solution, does not alter the clamping currents obtained with different anions in the outer solution. This shows that a cellular sodium current does not contribute significantly to the outward clamping currents. The ranking order of the halides with respect to carrying current through the skin, therefore, is the same as that obtained when comparing their exchange capabilities.

The positive clamping currents observed in a few cases in Table II occur when the spontaneous potential difference in iodide Ringer's is more negative than the preselected clamping voltage.

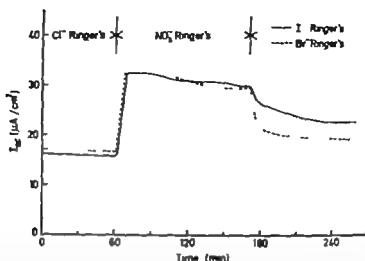


Fig. 1 Response of short circuit current to changes of the anion of the outside bathing medium. Two laterally symmetrical half skins were used. The outside anion was changed from chloride, to nitrate, to bromide in the one half skin, and from chloride, to nitrate, to iodide in the other half skin. The simultaneously measured chloride effluxes of this and seven similar experiments are given in Table I.

therefore be interpreted as inhibition of the chloride exchange component. Any effect of various drugs on the shunt flux is easily detected by measuring the outflux towards chloride free outer solution in the presence of the drug. Such control flux measurements with sulphate Ringer's outside were performed in nearly all experiments, and the results will be given when they are considered essential.

External anion substitution

In the first series of expts. the chloride efflux was measured in short circuited skins exposed in consecutive periods to (1) Cl⁻ Ringer's, (2) NO⁻ Ringer's, and (3) Br⁻ Ringer's or I⁻ Ringer's, see Fig. 1. After completion of the flux measurements of each of the three periods the skins were hyperpolarized for 5 min. which is sufficient to attain the steady-state clamping current.

The results concerning the chloride effluxes under short circuit conditions are presented in Table I. With nitrate Ringer's on the outside, exchange diffusion of chloride is absent.

TABLE I The effect on chloride efflux of changing the anion of the outside bathing solution. The skins are short circuited, and the inside is bathed with chloride Ringer's.

Exp. no	Chloride efflux (neq. cm ⁻² min ⁻¹)			
	Cl ⁻ Ringer's	Br ⁻ Ringer's	I ⁻ Ringer	NO ⁻ Ringer's
1	6.2	2.5	0.89	1.00
2	4.8	2.6	0.74	0.70
3	11.4	3.8	0.54	0.35
4	5.6	3.0	0.57	0.62
5	7.9	1.3	1.10	0.75
6	4.0	1.7	0.55	0.88
7	34.7	5.6	1.70	1.60
8	1.7	1.4	1.20	0.91

TABLE IV The effect of thiocyanate on chloride influx and simultaneously recorded clamping current in skins clamped at -80 mV. The skins used are the laterally symmetrical halves of those used for efflux measurements given in Table III. Concentration of SCN^- is 10 mM on both sides.

Exp. no.	Chloride influx $\text{meq. cm}^{-2} \text{ min}^{-1}$		Clamping current given as equivalent ionic flux ($\text{meq. cm}^{-2} \text{ min}^{-1}$)	
	Control	With SCN^-	Control	With SCN^-
1	21.4	10.7	33.3	9.8
2	37.3	26.5	44.4	20.5
3	60.5	36.7	69.3	42.2
4	7.7	8.8	10.2	2.1
5	16.6	9.3	22.2	7.5

The effects of thiocyanate on chloride influx and simultaneously recorded clamping current during hyperpolarization are presented in Table IV. It appears that both parameters are reduced considerably by the addition of SCN^- .

The effect of thiocyanate on the short circuit current recorded during chloride efflux measurements, is exemplified in Fig. 3 showing that the short circuit current is increased in the presence of 10 mM SCN^- in both bathing solutions.

Furosemide

Furosemide has been shown to inhibit chloride transport in the ascending limb of Henle's loop in the rabbit kidney (Borg *et al.* 1973). Table V shows the effect of this drug on chloride movements across toad skin. It can be seen that chloride efflux is reduced significantly, the remaining steady state efflux being only a little larger than the efflux usually measured with sulphate Ringer's on the outside.

In the hyperpolarized skin, furosemide reduces chloride influx and outward clamping current to about 50% of the respective values observed in the control period (Table V).

Fig. 4 summarizes the effect of furosemide on the short circuit current in the six experiments presented above. A small but transient increase of this parameter is observed.

Phloretin

The effect of phloretin added simultaneously to inside and outside bathing solutions is presented in Table VI. In four out of the 5 expts., a significant chloride exchange diffusion

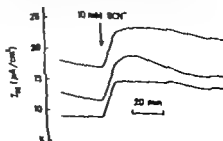


Fig. 3 Three examples of short circuit current recordings showing the effect of SCN^- (10 mM, both sides).

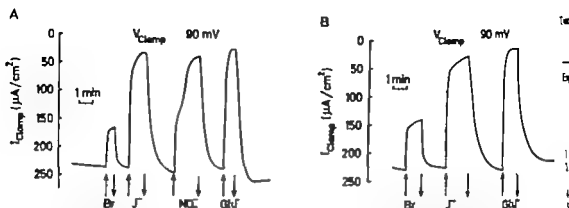


Fig. 2. An experiment showing the changes in clamping current obtained by sequential changes of the anion in the outside bathing medium, in the absence (A), and in the presence (B) of 60 μ M amiloride.

Inhibition by thiocyanate

Thiocyanate was shown to inhibit chloride transport in the frog skin (Kristensen 1972). Therefore the effect of this ion on chloride movements and clamping current in the toad skin was investigated. The effect of 10 mM SCN⁻ on the apparent exchange diffusion under short circuit conditions and on chloride influx under hyperpolarization was studied on skins from the same animal, one half skin being used for efflux measurements, the other for measurements of influx and clamping current. Table III shows the effect of SCN⁻ on chloride efflux. In some skins, the efflux measured in the absence of the inhibitor is already rather small which is often the case during the winter months. But by comparing these chloride effluxes with those obtained with sulphate Ringer's on the outside, it is possible to obtain a measure of the apparent exchange flux of chloride which is equal to the decrease in chloride efflux caused by changing the anion in the outside bathing medium from chloride to sulphate. Thus, by comparing columns 4 and 6 of Table III it can be seen that SCN⁻ inhibits chloride exchange diffusion across the toad skin.

TABLE III The effect of thiocyanate on chloride efflux under short circuit conditions. The apparent chloride exchange diffusion is obtained by subtracting the efflux measured with sulphate Ringer's on the outside from that measured with chloride Ringer's on the outside. SCN⁻ was added as NaSCN to a final concentration of 10 mM on both sides.

Exp. no.	Chloride fluxes (nec cm ⁻² min ⁻¹)				
	Efflux measured with chloride outside	Efflux measured with sulphate outside	Apparent chloride exchange diffusion without SCN ⁻	Efflux measured with SCN ⁻ added	Apparent chloride exchange diffusion with SCN ⁻ added
1	1.2	0.66	0.54	0.74	0.08
2	4.0	0.70	3.30	2.50	1.80
3	11.1	1.00	10.1	5.00	4.00
4	1.2	0.53	0.67	0.52	0.0
5	0.9	0.53	0.37	0.51	0.0

VI The effect of phloretin (0.1 mM on both sides) on chloride efflux under short circuit conditions, on clamping current during hyperpolarization, and on spontaneous transperithelial potential difference. Short circuit current values were obtained from the automatic recordings of the current during chloride efflux measurements. Measurement of chloride efflux into sulphate Ringer's gives estimates of the free diffusion sheet.

19	Chloride efflux $\mu\text{eq cm}^{-2} \text{ sec}^{-1}$		Clamping current $\mu\text{A cm}^{-2}$ (clamping voltage -110 mV)		Spontaneous PD mV		Short circuit current $\mu\text{A cm}^{-2}$		
	Sulphate Ringer's outside	Chloride Ringer's outside Control	Chloride Ringer's outside Phloretin	Control	Phloretin	Control	Phloretin	Control	Phloretin
	0.94	3.3	3.1	-91.4	60.0	-17	-39	10.8	9.1
	0.86	2.8	1.6	-37.1	-31.4	-21	-38	6.4	6.4
	0.65	3.5	1.4	40.7	-25.0	-31	-34	8.3	6.5
	1.71	2.0	2.3	-17.9	20.0	-30	-32	11.6	7.1
	2.16	8.8	9.7	-171.4	-21.4	-14	-25	12.1	11.3

In Fig. 3 hyperpolarization current is plotted against the chloride efflux measured under short circuit conditions. The results are divided into 2 graphs. On the right for clamping voltages between -100 mV and -110 mV and on the left for clamping voltages from -70 mV to -80 mV. In both cases a highly significant correlation is observed, although the scatter appears to be more pronounced at higher clamping voltages.

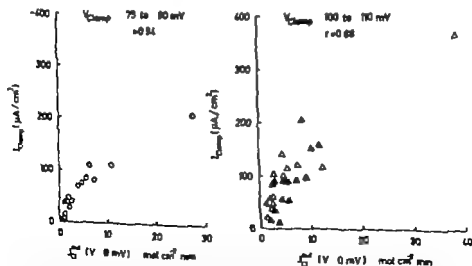


Fig. 3 The relation between chloride exchange diffusion and clamping current. Each point represents an experiment in which chloride efflux and hyperpolarization current were measured on the same skin preparation. The abscissa gives the total efflux, not corrected for the contribution made by the extracellular short pathway. Each normally amounts to less than $1 \text{ meq. cm}^{-2} \text{ sec}^{-1}$. A correlation analysis showed that I_{clamp} , I_{sc} and J^0 are correlated. correlation coefficients, 0.94 and $r = 0.88$, respectively. hypothesis $p < 0.0005$ for each group.

TABLE V The effect of furosemide (2 mM on both sides) on chloride efflux under short circuit conditions and on clamping current and chloride influx under hyperpolarization (clamping voltage, 80 mV). Fluxes and currents are given in $\text{neq cm}^{-2} \text{ min}^{-1}$

Exp. no.	Chloride efflux under short circuiting		Chloride influx under hyperpolarization		Clamping current recorded during hyperpolarization	
	Control	Furosemide	Control	Furosemide	Control	Furosemide
1	27.7	2.4	118.0	69.1	131	68
2	6.4	1.4	54.1	22.6	69	16
3	1.8	1.1	44.1	13.0	56	12
4	13.5	1.4	72.1	33.7	76	30
5	15.8	3.9	77.8	36.5	87	33
6	5.3	0.9	53.4	25.5	61	23

is evident. In these expts., phloretin inhibits the efflux of chloride under short circuit conditions as well as the clamping current under hyperpolarization. In one experiment (no Table VI) these inhibitions are absent. In this skin the exchange component is rather small (compare chloride efflux measured with chloride and sulphate outside, respectively); the measured clamping current can easily be accounted for by a comparatively larger diffusion shunt pathway of this skin preparation.

The short circuit current is not affected by the presence of phloretin, it remained at control value for at least one hour after the addition of the drug.

In the 4 expts. in which chloride movements are affected by phloretin, an increase in spontaneous transepithelial potential difference is observed upon addition of the drug.

Correlation between exchange diffusion flux and outward clamping current density

It is evident from the experimental results that the chloride exchange diffusion pathway and the conductive chloride pathway have properties in common. An interrelationship between these two pathways may also be disclosed by a correlation analysis relating chloride effluxes (\approx exchange diffusion) to the steady-state hyperpolarization currents in all our expts. in which these two parameters were measured on skins from the same animal.

Strictly the correlation analysis ought to be based on maximum chloride conductance minus exchange diffusion flux. But, from the start, the expts. were not designed for this purpose, so conductance measurements were not performed.



Fig. 4 The effect of adding furosemide (2 mM both sides) on the short circuit current. Averaged short circuit current records from the expts. of Table V in which chloride efflux measurements were performed.

furosemide inhibits most of the efflux of chloride under short circuit conditions, the remaining fraction is only slightly larger than that found when the skin is bathed on the outside with sulphate Ringer's. A similar effect has been reported by Bevia *et al.* (1974) on both influx and efflux of chloride across the frog skin (*Rana temporaria*). In skins from the same species, Natorchin (1975) found that furosemide increases the transepithelial potential difference without affecting the short circuit current. The increase in potential difference was not seen with sulphate-Ringer's bathing the outside of the skin, so he concluded that furosemide acts on a chloride pathway. In our expts. furosemide was added to both sides of the skin, and in contrast to frog skin (Natorchin 1975), the toad skin responded with a transient increase in short circuit current. The furosemide inhibition of chloride efflux under short circuit conditions as well as of clamping current under hyperpolarization, however remained unchanged during the whole observation period after the onset of inhibition. The increase in short circuit current and inhibition of chloride transport, therefore, cannot be interrelated in a simple manner. With furosemide, a stimulation of short circuit current was also observed by Fulgraf *et al.* (1973), who likewise found that the amount of sodium in the skin exchanging with radioactive sodium in the outside medium was increased by furosemide (Aumann and Fulgraf 1975). They concluded that furosemide when added to the outside, acts by increasing sodium permeability of the outer barrier and when added to the inside, that of the serosal membranes. The overall cellular sodium concentration however was not changed in the presence of the drug. Thus, it is unlikely that inhibition of chloride fluxes is caused by changes of cellular ion concentrations, and we conclude that furosemide interacts directly with chloride transport mechanisms. This conclusion is in accordance with that of Burg *et al.* 1973 who studied the effect of furosemide on various parts of the rabbit kidney tubule, and showed that the only effect of this diuretic is an inhibition of the transcellular chloride transport across the thick ascending limb of Henle's loop.

The effect of phloretin on sodium transport in epithelia has not been studied extensively. It has been reported to be ineffective when added to the outside solution of the toad bladder (*Bufo marinus*), and to reduce sodium transport by about 30%, when added to the inside bathing solution (Levine *et al.* 1973). In our experiments, an effect on short circuit current was not observed when the drug was added to both bathing solutions simultaneously. Thus, in the toad skin, it is clear that phloretin cannot affect the active sodium transport pathway. With this conclusion in mind it is interesting to note that phloretin was found to increase the transepithelial potential difference. This confirms our view (Bruus *et al.* 1976) that the cellular chloride conducting pathway is responsible for the low spontaneous potential difference observed across toad skins bathed on the outside with chloride-Ringer's.

In toad skin acetazolamide reduced chloride efflux under short circuit conditions as well as clamping current during hyperpolarization (Bruus *et al.* 1976). Under the experimental conditions used, the short circuit current was not affected. Also in frog skin, the short circuit current was unaffected by acetazolamide (Ehrh 1971; Kristensen 1972). These results lead to the conclusion that the effects observed in toad skin are due to a direct interaction with chloride transport systems.

The mechanism of action of acetazolamide should be considered briefly. Since this drug

Discussion

The results presented very clearly show similar effects on chloride exchange diffusion and on transcellular anionic current as a result of drug application and anion substitution respectively. It is tempting, therefore, to consider whether the two types of chloride transfer are dependent upon a common membrane component. Such a possibility has been proposed for the chloride conductance and the chloride exchange diffusion in red cells (Vestergaard Bogind and Lassen 1974) based on pharmacological evidence. It was suggested again recently in the case of chloride transports in Ehrlich ascites tumor cells (Simonsen, Hoffmann and Sjöholm 1976). Before we discuss this hypothesis, however, it should be considered whether the observed effects on the two chloride transport mechanisms are direct ones or whether they are secondary *i.e.* dependent on effects on other pathways *e.g.* the active sodium transport.

Effect and specificity of ionic substitutions and of inhibitors

Anion substitutions and thiocyanate application significantly stimulate active sodium transport. The nature of these effects cannot be evaluated from the present study. In the bladder Singer and Civan (1971) found that anions stimulate sodium transport with increasing efficiency according to the following order $\text{Cl} < \text{Br} < \text{I} < \text{NO}_3 < \text{SCN}$. This is in agreement with our recordings of short circuit current during measurements of chloride effluxes. Although, SCN did not replace chloride, the effect on short circuit current was manifest. It is therefore likely that also in toad skin bromide, iodide, nitrate, and the cyanate increase the sodium permeability of the outward facing membrane, as suggested by Singer and Civan (1971). Under conditions of an increased sodium transport it is unlikely that the cellular sodium concentration and, hence, the cellular chloride concentration decrease (Voite and Hänni 1972). Therefore this cannot explain the reduction of chloride efflux seen with all other anions in the outside bathing solution. We conclude that bromide and iodide do not react with the exchange diffusion system as effectively as does chloride.

The clamping current measured under hyperpolarization with chloride Ringers in outside medium is carried predominantly by an inward flux of chloride. The hyperpolarizing current is significantly reduced by substituting chloride in the outer bath with bromide or iodide. These responses are independent of whether the cellular sodium pathway operates or is blocked by amiloride. The most obvious explanation of these results, therefore, is that the difference in halide currents reflects the abilities of these anions to enter and pass the conducting channels. Bearing in mind that the mobilities of chloride, bromide and iodide in water differ by less than 3%, the observation of large differences in halide currents makes it probable that not the hydrated but the bare ions are the kinetic entities of the charge transfer process.

Bray and Gunn (1976) showed that *furosemide* interacts primarily with the chloride transport mechanism in human red cells. However the mechanism of action of *furosemide* in amphibian epithelia is not clarified, and the effect of this drug cannot *a priori* be considered to be due to a primary action on chloride transport mechanisms. In our

these two modes of chloride transfer we suggest that the exchange process and the charge transferring process are both confined to the same type of membrane molecules which, in one state, serve chloride exchange diffusion and, in the other, conductive chloride transfer. Because of the selectivity of the conducting pathway it can be supposed that the channels are specific sites for chloride binding. In the case of chloride exchange diffusion, one would also expect the membrane molecules involved to have binding sites for chloride. For exchange transport to occur these sites must be exposed alternatively to the two sides of the membrane, and since the chloride efflux is a saturable function of outside chloride concentration of the outer solution (Brous *et al.* 1976), binding of chloride to the sites must be a prerequisite for the transfer process to occur. This may be achieved in many ways, which can not yet be distinguished from each other at least not in a complicated epithelium. The range of possibilities covers the classical mobile carrier, a rotating carrier conformational changes, and gate-like mechanisms. To change any of these systems from a non-conducting to a conducting state involves only that the system be transformed, so that binding of chloride is not a prerequisite for the respective molecular movements included in all models of exchange diffusion. In the language of the mobile carrier hypothesis, the chloride carrier can move chloride free across the membrane. Such a change may be conceived to be caused either directly by changes in clamping voltage, or by changes in concentration of ions interacting with *e.g.* a regulatory site. The present results do not allow any conclusion with respect to these problems. Neither is it possible to point out any reason for the quantitative differences observed with respect to the efficiency with which ion substitutes and inhibitors act on the two modes on chloride transport.

The correlation between the exchange diffusion flux measured under short circuit conditions and the outward clamping current obtained in the voltage range of full conductance activation does not necessarily imply a causal interdependence of these two variables. However the correlation is in agreement with the hypothesis of a common molecular equipment of the two pathways. Therefore, the results of the pharmacological and the ionic specificity studies considered together with the result of the statistical analysis bring strong support to the hypothesis that the chloride exchange diffusion and the charge transferring transport of chloride take place via common membrane components.

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is known to inhibit carboanhydrase activity it has been suggested that its inhibitory effect is what is assumed to be a $\text{Cl}^-/\text{HCO}_3^-$ exchange in fish gill epithelium (Maetz and Garcia Romeu 1964, Maetz 1971) and in amphibian skin (Garcia Romeu, Salibián and Peres Hernández 1969, Erlj 1971) is due to a reduction of endogenously produced HCO_3^- . A significant carboanhydrase activity confined to the "flask cells" in the upper epidermis of *Rana pipiens* has been demonstrated (Rosen and Friedley 1973). The equality of outward clamping current and inward transport of chloride rules out the possibility that a significant HCO_3^- outflux is associated with the conductive chloride influx through toad skin. The conclusion is supported by the fact that addition of acetazolamide reduces the outward clamping current and the inward flux of chloride to the same extent (Bruus *et al.* 1976). The observation that chloride influx and outflux are equal in the toad skin clamped to a reversed potential, together with the observation that the clamping current is now carried by an inward flux of sodium, provide the evidence that HCO_3^- does not significantly exchange with chloride (Bruus *et al.* 1976). Due to these findings it can hardly be assumed that a high level of carboanhydrase activity is required for these chloride transport mechanisms. Therefore, we suggest that acetazolamide interferes directly with these pathways at the membrane level. A direct interaction of acetazolamide with a membrane component has also been suggested in the case of the neutral NaCl uptake across the mucosal membrane in rabbit ileum (Nellans, Frizzell and Schultz 1975). Likewise, the carboanhydrase inhibitor sulphonamide has been shown to interact directly with the carboanhydrase independent Cl^-/Cl^- exchange in the membrane of ox red blood cells (Cooper and Motaib 1976).

Although the drugs used may also affect the short circuit current (presumably the active sodium transport) the above discussion leads to the conclusion that the results obtained are caused by interactions between the drugs and the two chloride pathways.

The relation between the chloride conducting pathway and the chloride exchange pathway

In the preceding paper (Hviid Larsen and Kristensen 1977) it was shown that the chloride conductance is a function of the transepithelial clamping voltage. After a stepwise increase in clamping voltage, the conductance is time dependent and reaches its new steady state in the course of minutes. By comparing the results obtained with the predictions of a computer model of the two-membrane hypothesis, we came to the conclusion that the voltage dependence of the chloride conductance could not be explained solely by changes in membrane potentials and in intracellular chloride concentrations effectuating a "Goldman rectification" but that changes in chloride permeability must take place. The increase in chloride permeability occurring after hyperpolarization could be a direct consequence of the change of a membrane voltage. Alternatively it could be caused by changes in the intracellular concentration of some ion interacting with a modifying site, thereby regulating the chloride permeability. Our experiments do not enable us to distinguish between these two possibilities, but in both cases the increase in chloride permeability can be visualized as formation or opening of "chloride channels" ("gating").

In this paper we have compared the exchange diffusion pathway to the pathway allowing conductive transfer of chloride ions. In order to account for the striking similarity

These two modes of chloride transfer we suggest that the exchange process and the charge transferring process are both confined to the same type of membrane molecules which, in one state, serve chloride exchange diffusion and, in the other, conductive chloride transfer. Because of the selectivity of the conducting pathway it can be supposed that the channels are specific sites for chloride binding. In the case of chloride exchange diffusion, one would also expect the membrane molecules involved to have binding sites for chloride. For exchange transport to occur these sites must be exposed alternatively to the two sides of the membrane, and since the chloride efflux is a saturable function of outside chloride concentration of the outer solution (Brous *et al.* 1976), binding of chloride to the sites must be a prerequisite for the transfer process to occur. This may be achieved in many ways, which can not yet be distinguished from each other at least not in a complicated epithelium. The range of possibilities covers the classical mobile carrier, a rotating carrier, conformational changes, and gate-like mechanisms. To change any of these systems from a non-conducting to a conducting state involves only that the system be transformed, so that binding of chloride is not a prerequisite for the respective molecular movements included in all models of exchange diffusion. In the language of the mobile carrier hypothesis the chloride carrier can move chloride free across the membrane. Such a change may be conceived to be caused either directly by changes in clamping voltage, or by changes in concentration of ions interacting with a regulatory site. The present results do not allow any conclusion with respect to these problems. Neither is it possible to point out any reason for the quantitative differences observed with respect to the efficiency with which ion substitutions and inhibitors act on the two modes of chloride transport.

The correlation between the exchange diffusion flux measured under short circuit conditions and the outward clamping current obtained in the voltage range of full conductance activation does not necessarily imply a causal interdependence of these two variables. However the correlation is in agreement with the hypothesis of a common molecular equipment of the two pathways. Therefore, the results of the pharmacological and the ionic specificity studies considered together with the result of the statistical analysis bring strong support to the hypothesis that the chloride exchange diffusion and the charge transferring transport of chloride take place via common membrane components.

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The effects of pindolol and sotalol on neuromuscular transmission are tested using intracellular micro-electrode recording of resting membrane potentials, miniature end-plate potentials and end-plate potentials, and recording of muscle contractions upon nerve stimulation of rat phrenic nerve-hemidiaphragm preparations. Both pindolol and sotalol reduced the amplitudes of spontaneous end-plate potentials and end-plate potentials in dose-dependent manner without significantly affecting their time-courses. Pindolol, but not sotalol, also increased the frequency of the miniature end-plate potentials and decreased the number of acetylcholine quanta released by nerve impulses. Neither of the drugs significantly affected resting membrane potentials of the muscle fibres or excitability of the motor nerve to electric stimulation.

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The beta-blocking agent propranolol inhibits neuromuscular transmission both by exerting postsynaptic curare-like effect (Lillebell and Røed 1971, Wernman and Witlicki 1971, Larsen and Teräsvilinen 1977) and by decreasing the number of transmitter quanta released from motor nerve endings by a nerve impulse (Larsen and Teräsvilinen 1977).

The possible effects of other beta-blocking drugs on neuromuscular transmission have not been studied with intracellular recording methods. The present study was made in order to compare pindolol and sotalol, which differ from each other in their pharmacological properties. Pindolol has an intrinsic sympathomimetic activity and a membrane stabilizing activity (Levy 1971), whereas sotalol is devoid of both of these effects (Lish *et al.* 1965).

Methods

A total of 49 Sprague-Dawley rats, weighing 190 to 270 g, were used in the experiments. The animals were anaesthetized with ether and decapitated. The left diaphragm muscle and the phrenic nerve were dissected and prepared further in continuously oxygenated bathing solution. The phrenic nerve-hemidiaphragm preparations were placed in recording chamber with fixed volume of 4 ml. The bathing solution had the following composition (in mmol/l): NaCl 135, NaHCO₃ 16.5, Na₂HPO₄ 1, KCl 5, CaCl₂ 2, MgCl₂ 1 and glucose 11. It is continuously equilibrated with mixture of 95 oxygen and 5 CO₂. The pH of 7.35-7.40. The fluid in the recording chamber is replaced at constant rate of 400 ml

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Methods

A total of 69 Sprague-Dawley rats, weighing 190 to 270 g, were used in the experiments. The animals were anaesthetized with ether and decapitated. The left diaphragm muscle and the phrenic nerve are dissected and prepared further in continuously oxygenated bathing solution. The phrenic nerve-benzodiazepine preparations were placed in recording chamber with fluid volumes of 4 ml. The bathing solution had the following composition (in mmol/l): NaCl 135, NaHCO₃ 16.5, Na₂HPO₄ 1, KCl 5, CaCl₂ 2, MgCl₂ 1, and glucose 11. It was continuously equilibrated with mixture of 93% oxygen and 5% CO₂ and had pH of 7.35-7.40. The fluid in the recording chamber was replaced at constant rate of 400 ml

per hour. The drugs were dissolved in the bathing solution and introduced into the recording chamber from another reservoir. The drugs used were pindolol (Sandoz Ltd., Switzerland) mixed with tartaric acid 2:1 and sotalol chlorhydrate (Lääke Oy, Finland). The preparations were usually allowed to equilibrate for 30 min in the drug-free solution (control solution) or in the solution containing either of the drugs (pindolol and sotalol solution) both before the recording was begun and after the solutions were changed.

Intracellular recording of resting membrane potentials (RMP), miniature end-plate potentials (MEPP) and end-plate potentials (EPP) was made with glass capillary microelectrodes filled with 3 M KCl as selected for resistances between 5 and 15 megohms. A suction electrode was used to stimulate the phrenic nerve with a Grass S4 stimulator with an SIU 4678 stimulus isolation unit. The potentials were amplified with a Mentor N-950 preamplifier and displayed on a Tektronix 5115 oscilloscope. The traces were photographed from the storage oscilloscope screen and measured from magnified negatives.

All the recordings were made at a bath temperature of 23°C, and only those fibres with an initial RMP of at least -65 mV and which did not depolarize more than 5 mV during the experiments were used. MEPP with a rise time of less than 1 ms were included in the statistics. The MEPP amplitudes were corrected for differences in the RMPs of individual fibres using -75 mV as the standard RMP (Katz and Thiesell 1957). About 50 MEPPs were counted from each fibre.

In studying EPPs the phrenic nerve was stimulated with supramaximal current pulses 0.02 ms in duration and the amplitudes of the EPPs were reduced below the threshold for action potentials either by increasing the concentration of Mg^{2+} with a concomitant reduction of Ca^{2+} concentration (see Rastad) or by adding $7-8 \times 10^{-4}$ g/ml d-tubocurarine to the solution.

The quantum content (m) in high- Mg experiments was calculated from about 120 successive EPPs from each fibre as $m = \ln(\text{number of impulses/number of failures})$ and as $m = \text{mean EPP amplitude/mean MEPP amplitude}$ (del Castillo and Katz 1954), after correcting the amplitudes for nonlinearity of response (Martin 1955). Only those fibres which gave at least a 90% correlation between the two estimates were included in the statistics in which the exact value of the latter formula was used. In the experiments where the quantum content was so high that no failures occurred, only the latter formula was used. In order to estimate the size of the quanta and the mean quantum content in the curarized preparations, 100 successive EPPs were recorded from each fibre and the EPP amplitudes were corrected for nonlinearity of response and differences in RMPs using the formula given by Elmquist and Quastel (1965): $EPP^* (\text{corrected EPP}) = EPP / (RMP - 15 \text{ mV} - EPP)$. The quantum size (q) was calculated as $q = (\text{variance of } EPP^*) / (\text{mean } EPP^*)$ (Elmquist and Quastel 1965) and the quantum content as $m = (\text{mean } EPP^*) / (\text{variance of } EPP^*)$ (m^{-1} coefficient of variation of EPP^* (Martin 1955)). To convert back to mV values, q was multiplied by $(RMP - 15 \text{ mV})$, (Elmquist and Quastel 1965). The stimulation frequency in all quantum content experiments was 1 Hz.

To study the excitability characteristics of the motor nerve (strength-duration relationship), the phrenic nerve was stimulated with pulses of varying strengths and durations and the contractions of the diaphragm were recorded electrically using the strain-gauge principle.

The hemidiaphragm was fixed with insect pins to the bottom of the recording chamber only at the costal margin. An elastic steel blade with Phillips PR 9833K/OGSE strain-gages cemented on both sides was attached by a small hook to the free membranous side of the diaphragm, and the contractions recorded with the strain-gages were amplified with Tektronix 12A preamplifier and fed into a Tektronix 5115 storage oscilloscope. Student's t -test was used to determine the statistical significance of the differences between the means.

Results

The mean of the resting membrane potentials (RMP) of 90 fibres from preparations treated with pindolol (10 to 100 mg/l) was 77.2 ± 0.8 mV (mean \pm S.E.) and that of 92 fibres recorded in sotalol solution (10 to 100 mg/l) was 77.1 ± 0.7 mV. Neither of these values differed significantly from the control value of 77.4 ± 0.7 mV (mean \pm S.E.) obtained from 90 fibres.

The effect of pindolol and sotalol on the amplitudes of the miniature end-plate potentials (MEPP) is shown in Fig. 1. Both drugs caused a dose-dependent reduction in the MEPP amplitudes.

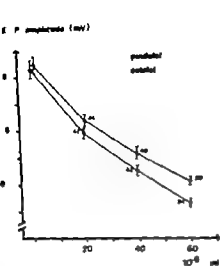


Fig. 1

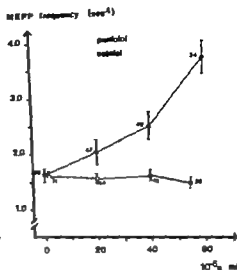


Fig. 2

Fig. 1 The figure illustrates the effects of pindolol and sotalol on the miniature end-plate potential (MEPP) amplitudes. A dose-dependent reduction is caused by both drugs. The points are the mean values of the number of fibres indicated by the small numbers adjacent to the points. Bars represent S.E. Ordinate: amplitude of the miniature end-plate potentials in mV. Abscissa: the concentration of the drugs.

Fig. 2 The effect of pindolol and sotalol on the frequency of the spontaneous release of transmitter (mean and S.E.). Pindolol increases the frequency, the effect being dependent of the concentration of the drug. No significant effect was observed with sotalol. Ordinate: the frequency of the miniature end-plate potentials (MEPP). Abscissa: the concentration of the drugs. The small numbers are the numbers of fibres studied.

The effect of the drugs began to manifest itself a few minutes after the beginning of the perfusion with either of the test solutions. The full strength of the effect was reached in 20 min. The drugs did not differ in this respect.

Fig. 2 shows the effect of the drugs on the frequency of the occurrence of MEPPs. Pindolol caused an increase in the frequency depending on the concentration of the drug, but sotalol had no significant effect. After washing the preparation for 30 min with the control solution, both the MEPP amplitudes and frequency were comparable to the controls, within an accuracy of at least 95%. The rise-time of the MEPPs recorded from 20 fibres in 40 mg/l pindolol solution was 0.52 ± 0.02 ms (mean \pm S.E.), and that of 22 fibres in 40 mg/l sotalol solution 0.51 ± 0.02 ms (mean \pm S.E.). Compared with the control value of 0.52 ± 0.0 ms (mean \pm S.E.), recorded from 26 fibres, no significant difference existed.

Both pindolol and sotalol caused a dose-dependent reduction of the end-plate potential (EPP) amplitudes in curarized preparations (Fig. 3). The effect was similar to that observed in the MEPP amplitude: the maximal effect was reached in 20 min and was reversible. The effect of pindolol was stronger than that of sotalol, the approximate concentrations needed to produce a 4-fold reduction of EPP amplitude compared with the control being 30 mg/l of pindolol and 45 mg/l of sotalol.

Both pindolol and sotalol (40 mg/l) significantly reduced the size of the quanta in

EPP amplitude
% of controls

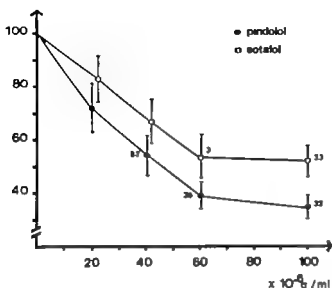


Fig. 3 The figure illustrates the dose-dependent reduction of the end-plate potential amplitude with pindolol and sotalol as percentage of the control. Mean and S.E. Ordinate: the mean amplitude of end-plate potentials (EPP) percentage of the control. Abscissa: the concentration of the drugs. The small numbers indicate the numbers of fibres studied.

curarized preparations (Table I). Neither of the drugs had significant effects on the time courses of the EPPs (Table I).

The effect of the drugs on the number of transmitter quanta released from the motor nerve endings by nerve stimulation was analyzed both in high-Mg-blocked (Mg^{++} 2 mmol/l and Ca^{++} 3 mmol/l; Mg 9 mmol/l and Ca 1 mmol/l) and in curarized preparations. Pindolol significantly reduced the mean quantum content in all three solutions tested (Table II). The effect was strongest in curarized preparations where the transmitter release was also greatest. No significant effect of sotalol on the transmitter release was observed (Table II).

The excitability of the motor nerve to electrical stimulation was studied by stimulating the phrenic nerve at a rate of one pulse per second and determining the weakest current strength sufficient to produce constant twitches of the diaphragm muscle at a fixed stimulus duration. No significant differences in the excitability of the nerve were observed either

TABLE I The effect of pindolol and sotalol on end-plate potentials in curarized muscle (Mean \pm S.E.)

	Amplitude (% of control)	Rise time (ms)	Half time (ms)	Quantum size (mV)
Control	100 (82) ^a	1.04 \pm 0.04 (15)	1.31 \pm 0.06 (15)	0.074 \pm 0.007 (24)
Pindolol (40 mg/l)	54.2 \pm 7.1 ^b (52)	1.03 \pm 0.04 (15)	1.29 \pm 0.06 (15)	0.013 \pm 0.001 ^b (24)
Sotalol (40 mg/l)	66.3 \pm 8.5 ^b (48)	1.03 \pm 0.04 (15)	1.31 \pm 0.05 (15)	0.015 \pm 0.01 ^b (26)

^a N = number of end-plates studied.
^b Significant at the level $p < 0.001$ compared with the control.

TABLE 1) The effect of pindolol and sotalol on the quantal content of end-plate potentials (mean \pm S.E.). A shows the quantal content recorded in a solution containing $7 \cdot 10^{-4}$ g/ml d-tubocurarine, B in a solution containing 23 mmol/l Mg^{2+} and 3 mmol/l Ca^{2+} and C in a solution containing 9 mmol/l Mg^{2+} and 1 mmol/l Ca^{2+} . The stimulus frequency was 1 Hz

	A	B	C
control	103.1 \pm 6.7 (14) ^a	9.28 \pm 0.30 (18)	1.31 \pm 0.04 (28)
pindolol (40 mg/l)	73.7 \pm 3.6 (25)	7.32 \pm 0.44 ^b (18)	1.17 \pm 0.03 ^b (29)
sotalol (40 mg/l)	101.9 \pm 6.1 (24)	9.29 \pm 0.52 (19)	1.27 \pm 0.04 (28)

Number of end-plates studied.

Significant at the level $p = 0.005$ compared with the control.

Significant at the level $p = 0.01$ compared with the control.

in the presence of pindolol (40 to 100 mg/l) or sotalol (40 to 100 mg/l) using pulse durations of 0.1 to 0.10 ms. The threshold current strengths for both drugs were kept within $\pm 2\%$ of the control values.

Discussion

It is evident from the present results that both pindolol and sotalol inhibit neuromuscular transmission by reducing the amplitudes of MEPPs and EPPs in a dose-dependent manner. Pindolol also reduces the number of acetylcholine quanta released from the motor nerve endings by nerve impulses, even though it slightly increased the frequency of the spontaneous release of MEPPs. Neither of the drugs seems to affect the rise-time or the half decay time of MEPPs and EPPs or the excitability of the motor nerve to electrical stimulation. That the reduction of the amplitudes of the MEPPs and EPPs is postsynaptic, and not due to a diminished amount of acetylcholine within quanta, or a depletion of the transmitter stores, is suggested by the rapidity and reversibility of action of the drugs. As it is dose-dependent and not combined with any changes in the time courses of the potentials, the postsynaptic action of the drugs is likely to be competitive inhibition resembling that of the curare effect.

In addition to the postsynaptic actions, pindolol had presynaptic effects on the transmitter release: it increased the frequency of MEPPs at the resting stage and diminished the release induced by nerve impulses. The first type of action of pindolol may be due to the intrinsic sympathomimetic activity of the drug similar to that of adrenaline, causing an increase in the MEPP frequency (Kuba 1970). Judging from the significances of the differences compared with the control, the postsynaptic effects of pindolol seem to be quantitatively more important than the presynaptic actions in the impulse transmission at the motor end-plate.

The depression of the quantal release of the transmitter upon nerve stimulation was most marked when the quantum content was high. It diminished proportionally when the mean quantum content was reduced by changing the ratio of Mg^{2+} and Ca^{2+} ions. This

seems to exclude the possibility that pindolol blocks a constant part of the population of transmitter quanta available for release. The reduction by pindolol of the number of acetylcholine quanta released may be due either to a reduced depolarization of the motor nerve endings or to a diminished probability of release in response to depolarization, or both.

Compared with the previously reported effects of propranolol on the neuromuscular transmission (Larsen and Teräsväinen 1977) the actions of pindolol and sotalol at the postsynaptic site were qualitatively similar. At the presynaptic site, however, important differences were observed. Propranolol and pindolol, which have a membrane-stabilizing effect (Morales-Aguilera and Vaughan Williams 1965; Levy 1971), were observed to depress the stimulus-induced transmitter release. Sotalol, which is devoid of any marked membrane effect (Lish *et al.* 1965), had no significant effects on the transmitter release. On the other hand, pindolol, which is the only one of these drugs with an intrinsic sympathomimetic activity, increased spontaneous transmitter release.

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Cardiovascular reactivity and design in rats with experimental "neurogenic hypertension"

By

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Abstract

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The arterial baroreceptors are desensitized in 20 normotensive Wistar rats. 2 1/2 months after the operation their cardiovascular responses to "mental stress" are compared to those of matched control rats (NCR). At the time of investigation the blood pressure of the baroreceptor desensitized rats (BDR) is increased some 15 per cent above that of the control group. There was, however, no difference in response to mental stress between groups, if anything the BDR responded with less pronounced tachycardia. Two months subsequent to the stress-test the mean blood pressure of the BDR was still significantly above normal levels, but the BDR did not exhibit structural cardiovascular adaptations, which has been documented in most types of stable hypertension as response to the increased mean blood pressure. The results imply that hypertension induced by baroreceptor denervation is not of stable and persistent type in which case structural changes in the cardiovascular system would have developed within the observation period (approximately 4 months). Endothelial stretch, however, do not seem to contribute to periodic blood pressure increases at the time of stress-testing, as earlier suggested.

Following their description by Hering in 1923 the systemic arterial baroreceptors were extensively investigated as a possible source of the aetiology of essential hypertension in man. It soon became apparent that section of the buffer nerves did not produce a model of essential hypertension but rather that a fluctuating blood pressure elevation and tachycardia resulted from this procedure (cf. Heymans and Noll 1958). Recently Cowley, Liard and Guyton (1973) have challenged even this assertion and have postulated that the arterial baroreceptors have little or no role to play in determining mean blood pressure integrated over the 4 h, but instead operate in such a manner as to oppose acute changes in blood pressure around the mean, at least in the dog. In other words, the baroreceptors do indeed function as buffer nerves in such a way that they not only minimise rises in blood pressure but they also lessen falls in blood pressure. According to these authors, the mean blood pressure itself is determined by other factors, possibly renal in origin.

seems to exclude the possibility that pindolol blocks a constant part of the population of transmitter quanta available for release. The reduction by pindolol of the number of acetylcholine quanta released may be due either to a reduced depolarization of the motor nerve endings or to a diminished probability of release in response to depolarization, or both.

Compared with the previously reported effects of propranolol on the neuromuscular transmission (Larsen and Teräsvälinen 1977) the actions of pindolol and sotalol at the postsynaptic site were qualitatively similar. At the presynaptic site, however, important differences were observed. Propranolol and pindolol which have a membrane-stabilizing effect (Morales-Aguilera and Vaughan Williams 1965; Levy 1971) were observed to depress the stimulus-induced transmitter release. Sotalol which is devoid of any marked membrane effect (Lish *et al.* 1965) had no significant effects on the transmitter release. On the other hand, pindolol which is the only one of these drugs with an intrinsic sympathomimetic activity, increased spontaneous transmitter release.

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allowed to recover in 3 months. catheter was placed in the caudal artery and the blood pressure recorded throughout the operation which usually took about 30-40 min to perform. In all 5 animals heart rate and pressure increased as a result of these manipulations. The rats were left for 2 1/2 months and then subjected to standardized "mental stress" test (cf. Halbläck and Follow 1974).

On the day of the "mental stress" test the animals are anesthetized briefly with ether and cannulae regarding blood pressure and heart rate are inserted into the tail artery. The rats are always studied pairs with one hypertensive determined rat (BDR) and one normotensive control rat (NCR). Local anesthesia is infiltrated around the base of the tail to minimize irritation from the site of insertion of the cannula.

The rats were then placed in a specially designed box with two identical chambers. Details of the stressing procedure have been published previously (Halbläck and Follow 1974). The stress stimuli consisted of the random playback (30 s) of either disturbing sound or vibration, after an initial acclimatization to the new environment of one hour's duration. 15 min recovery periods between the two types of stress were allowed. Some stress always preceded stress produced by vibration. Finally the animals were left for another period of 1 h so that the blood pressure could be monitored for a further period during which the variability in blood pressure is determined. This was done by counting each deviation greater than 5 mmHg from the mean blood pressure that occurred during this hour. It was also of interest to see whether a longer period of acclimatization to the new environment would cause further stabilization of blood pressure in BDR.

At the end of this additional period of observation and during stable blood pressure and heart rate conditions, 1 g/kg of saralasin was injected intra-arterially. The subsequent effect of the acute rise in blood pressure on the heart rate was then recorded as a test of baroreceptor efficiency in comparison with the normotensive controls. After terminating the experiment, the tail artery cannula was removed under brief ether anaesthesia and the animals returned to the animal house for a further period of 1 1/2 months before a study of cardiac and vascular design was made by means of a bloodquarter perfusion technique. 15 NCR and 15 out of the original BDR were included in the study. These are selected on the basis of the BDR having the highest blood pressures at the time of stress testing.

The presence of a elevated blood pressure (as known during the period of measurement) was confirmed also at the time of the bloodquarter perfusion studies by insertion of cannula into the tail artery under brief ether anaesthesia and then allowing the animal to regain consciousness so that awake heart rate and blood pressure could be determined.

Pairs of rats that had been used together during the stress procedure (1 one NCR and one BDR) were again studied together using paired bloodquarter perfusion technique (cf. Follow *et al.* 1970). Oxygenated Tyrode solution containing 4 dextran as colloid substitute as used as perfusate. In all other respects the technique is as used by Follow *et al.* 1970 and the construction of the resistance curve is characteristic of the individual vascular beds as is described by these authors. Briefly during maximal dilatation, achieved by repeated slow injections of papaverine, pressure-flow curves were constructed for each pair of bloodquarters from the pressure-flow recordings. The pressure responses during constant flow (1 resistance responses) were graded doses of noradrenaline (NA) were obtained in each pair of rats. Resistance curves are then constructed for each pair of animals as log NA dosed perfusate on the abscissa and perfusion pressure in mmHg on the ordinate. From these curves could be calculated the resistance 1 maximum dilatation, the threshold dose of noradrenaline which is defined as the noradrenaline concentration required to raise resistance 25 per cent above that at maximal dilatation, the steepness of the curve as well as the maximum curve response which is induced by large doses of noradrenaline (10 IU) or barium chloride, 150 mg. The mean S.E. for these points are calculated for both groups of rats and the differences between the BDR group compared to the NCR group evaluated by means of the paired design t-test.

The hearts are removed from all the animals and the left ventricles dissected free and weighed. Then the mean left ventricular weight is calculated as was the percentage left ventricular weight/body weight and mean value of the two groups compared.

Results

In a preliminary test four rats died during the operation or immediately thereafter. With slight modification of the operation technique 20 operated rats all survived the operation. 15 of these BDR were studied further on the arbitrary basis of their higher blood pressures as measured after one hour of acclimatization in the stress box but before the first period of

In support of this hypothesis it has been found that the arterial baroreceptors are essential in essential and renal hypertension to operate around the higher level of mean arterial pressure (cf McCubbin, Green and Page 1956 Angell James 1973). However this resetting may be largely a secondary phenomenon related to structural vascular changes which are a consequence of the high blood pressure (Angell James 1973 Jones 1976).

Cowley *et al* (1973) found that alerting stimuli caused great elevations of blood pressure in their baroreceptor denervated dogs and suggested that such manoeuvres as measure the blood pressure in experimental animals usually amounted to alerting stimuli. It was surprising, therefore, that earlier investigated baroreceptor-denervated animals were almost invariably found to be hypertensive and to have tachycardia. By avoiding such stimuli it was found that during 24 h blood pressure recordings in baroreceptor denervated dogs, the peaks and troughs in blood pressure levels occurred so that the mean blood pressure over the 24 h was little different from that of control animals.

The troughs in blood pressure occurring with such a preparation may however at least in part be due to chemoreceptor and not to baroreceptor denervation (Zanchetti, Ge and Baccelli 1967). Under these circumstances, therefore, if the denervation were limited to baroreceptors only (a technically difficult thing to achieve) an elevation in mean blood pressure might result. Furthermore, sustained "hypertension" results in man after carotid endarterectomy (Wade *et al* 1970) and after extirpation of the carotid body (Sleight 1967) a manoeuvre that is almost certain to damage the sinus nerve as it courses over the carotid body.

"Neurogenic hypertension" by buffer nerve section has been produced successfully in the rat (Krieger 1964, 1967 Thant, Yamori and Okamoto 1969). Krieger (1964) found a sustained elevation of blood pressure and tachycardia persisting for the duration of his study (over 1 year), but the question again arises to what an extent the procedures used during measurements might have evoked alerting responses and therefore transient pressure elevations. Significantly however there was here histological evidence of left ventricular hypertrophy and also minor changes in the basement membranes of the renal glomeruli in these rats.

The present study was undertaken to try to clarify some of these apparently contradictory results. In order to explore if baroreceptor denervation causes an increased cardiovascular reactivity to alerting stimuli rats were exposed to a standardized "mental" stress 2 months after denervation of the arterial baroreceptors. A further 2 months later the rats were investigated by means of a hemodynamic analysis to explore if structural changes in the cardiovascular system had occurred as an adaptive response to an increased mean arterial pressure, as previously has been shown in other types of chronic hypertension (cf Folk *et al* 1973).

Methods

20 male Wistar rats weighing approximately 200 g and aged 7 weeks were subjected to a slight modification of Krieger's (1964) "standard" technique for the production of neurogenic hypertension in the rat. The procedure was briefly that of section and removal of a length of both superior laryngeal nerves and both cervical sympathetic trunks under ether anaesthesia. This resulted in denervation of the aortic baroreceptors. The carotid sinuses were carefully cleared of all connective tissue and then palpated with a phenol in alcohol as extensively as possible resulting in their denervation also. The incision in the skin was sutured and infiltrated with local anesthetic after which the ether was discontinued and the animal

owed to recover 15 animals. catheter was placed in the caudal artery and the blood pressure recorded throughout the operation which usually took about 30-40 min to perform. In all 3 animals heart rate and blood pressure increased as a result of these manoeuvres. The rats are left for 1/2 months and then subjected to standardized "muscular stress" test (cf Halbläck and Folkow 1974).

On the day of the "muscular stress" test the animals were anaesthetized briefly with ether and cannulae recording blood pressure and heart rate are inserted into the tail artery. The rats were always studied pairs: one baroreceptor denervated rat (BDR) and one normotensive control rat (NCR). Local anesthetic was infiltrated round the base of the tail to minimize irritation from the site of insertion of the cannula.

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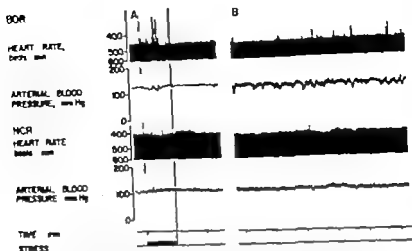
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The present study was undertaken to try to clarify some of these apparently contradictory results. In order to explore if baroreceptor denervation causes an increased cardiovascular reactivity to alerting stimuli rats were exposed to a standardized "mental" stress month after denervation of the arterial baroreceptors. A further 2 months later the rats were investigated by means of a hemodynamic analysis to explore if structural changes in the cardiovascular system had occurred as an adaptive response to an increased mean arterial pressure, as previously has been shown in other types of chronic hypertension (cf Folkow *et al* 1973)

Methods

20 male Wistar rats weighing approximately 300 g and aged 7 weeks were subjected to a slight modification of Krieger (1964) standard technique for the production of neurogenic hypertension in the rat. The procedure was briefly that of section and removal of a length of both superior laryngeal nerves and both cervical sympathetic trunk under ether anaesthesia. This resulted in denervation of the arterial baroreceptors. The carotid sinuses were carefully cleared of all connective tissue and then painted with 10% phenol in alcohol as extensively as possibly result of the denervation also. The incision in the neck was sutured and infiltrated with local anesthetic after which the ether was discontinued and the animals



1 Part A shows the actual heart rate and blood pressure traces from one baroreceptor denervated rat (BDR, upper two recordings) and from one matched normotensive control rat (NCR, lower two recordings) which were simultaneously investigated in standardized "stress-test". The stimulus applied was in this case exposure to violent vibrations. Part B is continuous 5 min recording of blood pressure and heart rate of the two rats. Note the greater lability in blood pressure in the BDR.

Variability in basal blood pressure was decidedly more marked in BDR than in NCR. During the 1 h observation following the periods of stress blood pressure deviated more than 5 mmHg from baseline 240 ± 21 times in BDR while the equivalent figure for NCR was 142 ± 19 ($p < 0.001$). The great majority of these deviations from resting pressure were, however, falls in blood pressure (see Fig. 1).

As a test of baroreceptor function the reflexogenic bradycardia in response to an angiotensin-induced blood pressure increase was investigated. During the rapid initial blood pressure rise, an increase of 25 mmHg caused the heart rate to fall 25 ± 4 beats per min in the NCR. No change at all, or 0.1 ± 5 beats per min, was observed in the BDR indicating that BDR were efficiently deprived of their arterial baroreceptor control.

At the time of the stress test the body weight of the BDR was 333 ± 6 g and that of the NCR was 343 ± 3 g and hence there was no difference in body weight. 6 weeks elapsed until the hemodynamic analyses were performed during which time the NCR increased in weight by 58 ± 5 g while the BDR only gained 34 ± 4 g. These differences in weight gain were significantly different ($p < 0.01$) and, therefore, the NCR were significantly heavier (399 ± 7 g) than the BDR (370 ± 6 g) when the hemodynamic analysis was performed ($p < 0.01$).

The hemodynamic analyses were performed on 15 pairs of BDR and NCR. During simultaneous perfusion of the two hindquarter vascular beds in each pair there was no difference between BDR and NCR in resistance during maximal vasodilatation, i.e. when all vascular smooth muscle activity was abolished (see Table I). Sensitivity to noradrenaline was equal in the two groups as judged by equal threshold doses. The slope of the resistance curves of the two groups did not differ substantially and no difference in maximal pressure response was observed between the two groups (see Table I). Thus, as judged by these para-

TABLE I Gives the mean values \pm S.E. for mean arterial blood pressure and heart rate for 15 baroreceptor denervated rats (BDR) and for 15 normotensive control rats (NCR) at the time of "stress-test". Furthermore from the hemodynamic analysis are presented the resistance at maximal vasodilation at a flow of 20 ml/min 100 g, the steepness of the resistance curve and the maximal pressure response. Left ventricular weight in per cent of body weight is also presented for the two groups. Levels of significant difference between groups are also given.

	Mean arterial blood pressure, mmHg	Heart rate, beats/min	PRU ₁₀₀ at max. dil. Q = 20 ml/min 100 g	Tangent of the angle of the resistance curve	Maximal pressure response mmHg	Left ventricular weight in per cent of body weight
BDR n = 15	126 \pm 2	419 \pm 7	2.4 \pm 0.1	2.5 \pm 0.3	219 \pm 11	0.177 \pm 0.004
NCR n = 15	111 \pm 2	371 \pm 8	2.5 \pm 0.1	2.6 \pm 0.4	209 \pm 14	0.174 \pm 0.004
Significance p <	0.001	0.001	0.5	1.0	0.5	0.5

stress (mean arterial blood pressure 126 \pm 2 mmHg, see Table I). These 15 rats were considered to have "neurogenic hypertension". Mean arterial blood pressure in the control group of rats was 111 \pm 2 mmHg, which was significantly lower than that of the BDR ($p < 0.001$). Thus the BDR had a mean blood pressure increase of 15 mmHg or 14% over the NCR at the time of recording. Just prior to the hindquarter perfusion study the BDR had a mean arterial blood pressure of 126 \pm 2 mmHg and their matched NCR 107 \pm 3 mmHg before anesthesia was given. Neither of these values, recorded in the awake rats, had changed significantly when compared to the same 15 pairs at the time of stressing. Mean arterial blood pressure after administration of nembutal but before the operative procedures were started were 125 \pm 2 in BDR and 104 \pm 3 in the NCR. There was hence no significant difference in mean blood pressure in either group after the barbiturate had been applied.

"Basal" values of blood pressure and heart rate were obtained in the awake rats after one hour's accommodation in the stress box in a semi-dark, soundproof room. At this time the rats had recovered from the superficial ether anesthesia required for insertion of the tail artery cannula. The mean changes in heart rate and blood pressure during the 30 s of stress and also during the succeeding 30 post stress seconds were obtained in the following way. The changes in heart rate and blood pressure from prestimulatory value were calculated for each third second in each rat, after which the difference in response between the paired BDR and NCR at each third second interval was calculated. The mean of these paired differences, and the standard errors of the mean differences, were then calculated as well as the significance differences in response (cf. Hallbäck and Folkow 1974). A significant difference in total response was considered to be present when 6 out of the 10 observations during stress stimuli or 12 of the total 20 observations, differ above 95% probability.

In this way it was found that the neurogenically hypertensive rats responded with less pronounced tachycardia than did the NCR. This may be due to the higher resting heart rates in the BDR. However both groups preferentially responded with tachycardia in the stress period so that there was no difference between the BDR and NCR as to the direction of response during the stress stimulus. The blood pressure responses during stress were of equal magnitude in the two groups.

ontaneously hypertensive rat (MHS) showed a good correlation between the degree of left ventricular hypertrophy and the elevation of mean arterial blood pressure even though arterial blood pressure in these MHS was only elevated some 15 per cent (Hallböök *et al.* 1976), as in the present BDR at the time of measurement. In rats, the structural adaptation of the left heart and precapillary resistance vessels is a rapid process being practically complete within 2-3 weeks of hypertension, when induced by renal artery obstruction (Lundren *et al.* 1974). The presently used BDR were denervated for between 3-4 months. There are structural changes should have been detectable within this time scale in case the measured increase in mean arterial pressure of 15 per cent really represented the average increase throughout the 24-h during these 4 months. It would seem therefore that the mean blood pressure as here recorded in the awake animals, either before stressing or just prior to a hemodynamic analysis, is not a true reflection of the average pressure level over 24 h. The absence of the structural changes within the cardiovascular high-pressure compartments in the present BDR model strongly suggests that their average level of mean arterial pressure is not elevated over 24 h.

Following baroreceptor denervation in dogs Cowley *et al.* (1973) found wide swings in blood pressure thought to be associated with alerting stimuli. In particular the entry of the log attendants into the room caused tachycardia and acute rises in blood pressure. After a few months, these rapid swings in pressure were less marked but still considerably more pronounced than in control dogs. Many such oscillations in the pressure occurred for apparently no external reason, i.e. without obvious alerting stimuli though there might of course, have been such stimuli that were experienced only by the animals and not by the experimenter. We observed similar more frequent pressure oscillations in BDR compared to NCR and many of these occurred when the animal appeared to be peaceful at rest. However the majority of these pressure deviations from baseline were in fact *reflections* of blood pressure, and this alone might over a longer time period account for largely unchanged average pressure over 24 h in BDR.

During the standardized periods of stress neither BDR nor NCR showed any marked blood pressure responses and BDR were in this respect not hyperreactive, contrary to the findings in dogs by Cowley *et al.* (1973). This difference between species might be due to a rather stimulus being applied in the present study which however seems unlikely since the standardized stress stimuli used induce pronounced blood pressure increases in the spontaneously hypertensive rat (SHR) (Hallböök and Folkow 1974). If anything, the tachycardia response to stress was less in BDR than that in NCR, presumably related to the higher prestimulus heart rates in BDR as a result of baroreceptor denervation.

Thus, according to the present findings baroreceptor denervated rats do not respond differently than do normotensive control rats during stress stimuli. In this respect our results would, at first sight, seem to be at variance with those of Cowley *et al.* (1973) in dogs. The environmental stimuli used in our study were, of course, unpleasant in nature while those applied to the baroreceptor denervated dogs rather might have evoked pleasurable responses being induced when the animal keeper entered the experimental room. Hence the two types of stimuli would be entirely different in nature and are likely to induce different patterns of autonomic responses. When investigated during 24 h the neurogenically "hypertensive"

meters, *i.e.* resistance at maximal vasodilatation, the slope of the resistance curve and maximal pressor response, the resistance vessels of the BDR did not differ structurally from those of their normotensive controls. Structural adaptation of the left ventricle seem not to have occurred either since left ventricular weight in percentage of body weight did not differ between BDR and NCR being 0.177 ± 0.004 per cent *vs.* 0.174 ± 0.004 respectively ($p < 0.5$) (see Table I).

Discussion

The results of the present study largely support the contention of Cowley *et al.* (1973) that the term "neurogenic hypertension" may be a misnomer for animals deprived of their baroreceptor reflexes.

However a complete baroreceptor denervation is difficult to achieve and Krieger *et al.* introduced the technique for baroreceptor denervation in the rat, found only a 70 per cent success rate in achieving "hypertension" after one year. Furthermore a fairly high mortality was reported (Krieger 1964). Thant *et al.* (1969) have suggested that this was due to the acute chemoreceptor denervation in the anesthetized animal and were able to decrease mortality by not anesthetizing the animal during the operation. In the present study animals used for test operations developed respiratory difficulty and died all the more when all the nerves coursing near the carotid sinus were divided in order to clean the site for local phenol administration. If only these nerves were first displaced and not sectioned the animals survived the operation well. Thus, by a slight adjustment of Krieger's technique we were able to obtain 100 per cent survival and as much as 75 per cent of the operated rats had blood pressures greater than or equal to 120 mmHg.

The mean blood pressure of the 15 rats used was some 15 per cent above that of the NCR during the period of measurement. This increase in mean blood pressure may therefore be taken as a fairly good indication of successful baroreceptor denervation. Furthermore, the operated rats had increased heart rates when compared to their appropriate NCR and the 15 used for stress comparisons showed greater and more frequent fluctuations of blood pressure when compared to NCR (see Fig. 1). Finally angiotensin injected intraarterially caused an abrupt increase in blood pressure in both NCR and BDR but reflex slowing of heart rate occurred only in NCR, a response that is thought to be baroreceptor mediated (*cf.* Korner *et al.* 1974). Taken together these observations indicate that the 15 operated rats used could be considered to be adequately baroreceptor denervated for the purposes of the present study.

The present results further show that in rats who have had their systemic arterial baroreceptors denervated up to 3 1/2 months previously no structural adaptive changes occurred in either the resistance vessels or in the left ventricle. This is despite the fact that these rats had on the two occasions when pressure was measured approximately a 15 per cent increase in mean arterial blood pressure compared with matched paired normotensive control rats. Structural cardiovascular changes have repeatedly been found in chronic hypertension, both in human essential hypertension and in spontaneously hypertensive rats and in secondary renal hypertension, and to an extent that is largely proportional to the increase in mean arterial blood pressure (*cf.* Folkow *et al.* 1973). Furthermore, a recent study on the MII

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dogs as a group displayed a diurnal variation in blood pressure with variations as great as 15 mmHg. The peaks in pressure did, however, not appear to be associated with the surrounding noise level, as would be expected if "noxious" stimuli are particularly apt to elicit greater pressure responses in baroreceptor denervated animals. Attempts were made in the present group of rats to follow mean arterial pressure during 24 h but for technical reasons they were unsuccessful due to the small size of the animal which makes it particularly difficult to leave them really undisturbed. In the baroreceptor denervated dog the lower blood pressures were recorded in the early hours of the morning when the animals presumably were asleep (Cowley *et al* 1973).

Although Ferrario *et al* (1969) have failed to observe that blood pressure falls during sleep in baroreceptor denervated animals and they indeed noted an increase in blood pressure due to an increase in peripheral resistance, there is other evidence that mean arterial pressure falls markedly during deep sleep in such animals (Guazzi and Zanchetti 1969). It has then been suggested that a precipitous fall may only be prevented by activation of the peripheral arterial chemoreceptors (Zanchetti *et al* 1967). It is possible, therefore, that the BDR may have been relatively hypotensive when deeply asleep even though no difference in mean blood pressure was obtained after nembutal anesthesia. Although the animals were "hypertensive" during the time they were exposed to acute study the lack of adaptive structural changes in heart and resistance vessels strongly suggests that there were compensatory periods of hypotension keeping the averaged mean arterial blood pressure over 24 h in a normotensive range.

The observation that BDR did not gain weight as rapidly as NCR is of additional interest. They were fed the same diet and housed side by side under identical conditions as their NCR counterparts at least for the two months prior to the terminal experiment. They seemed to thrive as well as the NCR and showed no evidence of discomfort or poor health. The presence of intimate connections between the buffer nerves and the medial reticular formation implies that changes in baroreceptor activity may induce behavioural changes. It has thus been shown that powerful baroreceptor excitation inhibits sham rage (Bartorelli *et al* 1960) and may even induce signs of sleep (Bonvallet-Dell and Hiebel 1954). Therefore, chronic deactivation of the buffer nerves may perhaps induce an increased alertness or a restless behaviour in such a way that e.g. the balance between food intake and general metabolism is altered in BDR. This aspect of baroreceptor control merits, however, further study.

In conclusion we have shown, in agreement with Cowley *et al* (1973) that the "hypertension" consequent upon baroreceptor denervation seems not to be of a stable and persistent type in the sense that mean arterial pressure over a longer time period really is elevated. Had this been the case structural changes in the cardiovascular system would have developed within the time between operation and hemodynamic analysis as indicated by several other studies on rats with various types of mild or severe chronic hypertension. BDR do exhibit wider and more frequent oscillations in blood pressure when compared to NCR but the peaks, at any rate, do not necessarily seem to be evoked by overt alerting stimuli and transient pressure reductions are even more frequent than the pressure elevations. The arterial baroreceptors do indeed, seem to function as "buffer nerves" but seem to have little to play in setting of average level of mean arterial pressure throughout the whole day.

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Ultrastructure of synaptosomes from fetal rat brain

By

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Abstract

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The crude mitochondrial fraction P and subfractions of P were prepared from the brain stem, 1 sphaeres and whole brain of 19-day-old fetal rats. Samples were fixed in glutaraldehyde-osmium, Na₂ or by Trautner's triple fixation method (aldehyde-chromate-dichromate-osmium) and examined by electron microscopy. The C-fraction from whole brain was the main synaptosome fraction, consisting of 3.2% presynaptic terminals as counted from 11 membrane bound particles. The brain stem showed a presynaptic terminal to the hemisphere (2.8% versus 0.9%) suggesting a caudal-ventral motor gradient for synaptogenesis. The maturity of the nerve endings obtained was very variable in contrast to the rather uniform synaptosomes derived from adult tissue. They varied from profiles without any structures to mature synaptosomes displaying asymmetric synaptic junctions. Monoamine synapses containing small granular vesicles were not detected in the present study suggesting immaturity of granular monoamine pool at this stage of development.

Key words: Synaptosomes, development, ultrastructure, rat, brain

The application of cell fractionation techniques in studies of nervous tissue (see Whittaker 1965, 1972, Jones 1975) has resulted in significant progress in the knowledge concerning neuronal function. It is assumed that on homogenization the terminal region of the nerve is "pinched off" and seals up to form a discrete subcellular particle, which carries with it a length of the characteristically thickened postsynaptic membrane. Comprehensive reviews of the morphological and biochemical properties of pinched off nerve endings (synaptosomes) have been published (Whittaker 1955, 1972, Jones 1972, 1975).

Isolated nerve endings have also been used with success in studies on several aspects of the ontogenesis of the nervous system (Jones 1972, Tissari 1973, 1975). The number of nerve endings *in vivo* is small during early maturation (Tennyson 1970 a, Hervonen *et al.* 1974 a, b). Thus, the subcellular fractionation technique is especially suitable in studying the fine structure of the developing nerve endings, since large numbers can be obtained for examination with electron microscopy.

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With the exception of short notes on the fine structure of synaptosomes from fetal rat brains (Abdel-Latif and Abood 1964, Abdel-Latif *et al.* 1967) there is virtually nothing in the literature concerning development of synaptosomes at fetal stages in vertebrates. Furthermore, the results of Abdel-Latif and coworkers are in conflict with more recent observations. Abdel-Latif and Abood (1964) and Abdel-Latif *et al.* (1967) reported that maximal structural changes occurred between the 16th fetal and the first postnatal day at which stage the nature did not differ from that found in the 25-day-old and adult rat. Other workers (Jones and Revell 1970, Piras *et al.* 1970, Gonatas *et al.* 1971) found that synaptosomes formed during the first few postnatal days were very primitive and that maximal structural changes occurred considerably later.

We have recently confirmed and extended the results of Jones and Revell (1970) and Gonatas *et al.* (1971) in the 3-day-old rat (Hervonen *et al.* 1974, Kaferia *et al.* 1976, 1977). The purpose of this further study of the ontogenesis of synaptosomes was partly to analyze the fine structure of fetal rat synaptosomes. This is of importance in understanding the general events during synaptogenesis and in promoting a better basis for evaluating biochemical and pharmacological data in this age groups (Tissari 1973, 1975, Tissari and Ranno 1975). We also wanted to compare synaptosomes (synaptogenesis) from the brain stem and the hemispheres, since a caudal-rostral maturation gradient has been found for the brain content of several neurotransmitter constituents (McCartan and Aprison 1964, Porcher and Hoffer 1972, Coyle and Axelrod 1971, Tissari 1973). Finally we also made an attempt to demonstrate the granular pool of monoamines in the nerve endings at this early stage of development.

The results have been partially communicated in conference reports (Kaferia *et al.* 1975 b, c).

Material and Methods

Preparation of subfractions

19-day-old Sprague-Dawley rat brains were used in this study. Subfractions were prepared from the brain essentially as described by Rodriguez de Laver Arriaza and DeRobertis (1964), modified according to Tissari *et al.* (1969).

Brain tissue (whole brains, hemispheres, or brain stems) was homogenized in 9 volumes of 0.32 M sucrose containing 10^{-3} M CaCl_2 using glass homogenizer and Teflon pearls with approximately 0.25 inch clearance and 70 strokes. The homogenate was centrifuged at 1000 g for 10 min. The supernatant was removed and the pellet resuspended with 10 ml 0.32 M sucrose containing 10^{-3} M CaCl_2 . The pellet suspension was centrifuged at 1000 g for 10 min. The two supernatants were combined and centrifuged at 10000 g for 20 min. The supernatant was then decanted and the pellet (P or crude mitochondrial fraction) resuspended in suitable volume of 0.32 M sucrose with 10^{-3} M CaCl_2 (usually 5-10 ml) for layering on discontinuous sucrose gradient.

The crude mitochondrial fraction derived from 2.3 g of original tissue per gradient was resuspended in 0.32 M sucrose and layered over discontinuous sucrose gradient consisting of layers of 1.4, 1.0, 1.0 and 0.8 M sucrose. The gradient was centrifuged at 55000 g for 120 min in Spinco Model L ultracentrifuge equipped with SW 25.1 rotor. The fractions of sucrose gradient were removed by aspiration and diluted with enough water to produce sucrose concentrations of 0.32 M for subfractions A and B and 0.55 M for C, D and E. After standing for 20 min the suspensions were centrifuged for 30 min at 15000 g except for subfractions A and B which were centrifuged at 40000 g. Pellets were suspended in 14 ml of Krebs bicarbonate solution or other resuspension media, e.g. Na⁺-free Krebs solution containing all other electrolytes and with its osmolarity maintained by sucrose. Intact subfractions usually contained 0.2-0.3 mg/ml. Aliquots were removed for electron microscopy (see below).

Fixation for electron microscopy

The samples were fixed either as a suspension (glutaraldehyde, permanganate) or pellet (all the following fixatives were used (all concentrations final): (1) 3% glutaraldehyde in 0.1 M phosphate pH 7.4 for 120 min, (2) 6-9% sodium permanganate in phosphate buffer pH 7.4 for 45 min (Lippman 1966, Håkfelt *et al.* 1970) or (3) aldehyde-chromate-dichromate-osmium (triple fixation) and co-workers (Tranzer and Snipes 1968, Tranzer 1972, Tranzer and Richards 1976). The synapses were fixed as pellets in a mixture of glutaraldehyde (1%) and formaldehyde (0.4%) buffered to 0.1 M chromate-dichromate, for 30 min. The fixation was continued overnight in a 0.2 M dichromate solution at pH 6.0. The glutaraldehyde and aldehyde-chromate-dichromate fixed axons were postfixed in phosphate buffered 1% OsO₄ at pH 7.4 for 1-2 h. Then the specimens were dehydrated in Epon Araldite. The NaMnO₂-fixed sections were viewed and photographed directly. The other specimens were grid-stained with lead citrate and uranyl acetate. A Zeiss EM 9A electron microscope operated at 60 kV was used.

Incubation of synaptosomes with substrates

Samples for incubation with substrates were transferred to a Dubnoff water bath shaker and at 37°C and 95% O₂+5% CO₂ for 8 min before addition of substrate. After adding substrate, the incubation was continued and aliquots were removed after 15 or 30 min. Aliquots (2 ml, except A-type 3.5 ml) were removed to pre-cooled plastic tubes in an ice bath. After 20 min, samples were centrifuged at 10 000 g for 15 min and the pellet was fixed for EM. Substrates used were 5-hydroxy-dopamine or L-methyl-noradrenaline (α -methyl-NA). The substrate was used in a concentration of 100 added in 0.2 mg/ml ascorbic acid.

Quantitative electron microscopic calculations

For quantitative calculations, 5-10 non-overlapping serial electron micrographs were taken of pellets to insure their representative nature. A primary magnification of approximately 13 000 \times (16 500) was used. The negatives of these samples were then photographically enlarged ($\times 3$). Synaptic vesicles were readily recognized. Measurements of synaptic vesicles were made using a grid under magnifying glass. Two measurements were made on each vesicle (its maximum outside and a measurement at right angles to the long axis). The mean was considered the size of the vesicle.

To calculate the number of membrane-bound particles (MBP) with or without synaptic vesicles larger than 0.3 μ m in diameter with 3 or more vesicles 200-600 Å in diameter were characterized as synaptic endings.

Results

The best preservation was obtained with glutaraldehyde fixation in suspension by osmication. The description below mainly refers to the fine structure after this procedure. The difference in fine structure after different fixatives, the regional variations and the effect of incubations are described subsequently.

Fine Structure of the Fractions

Fraction P2

The main components were membrane bounded particles (MBP), free mitochondria, membrane fragments (devoid of associated cytoplasm) (Fig. 1-4). Some of the MBP's contained synaptic vesicles and some small intra-axonal mitochondria. They also represented developing or in a few cases mature pinched off nerve endings (synaptic terminals). Sometimes these synaptosomes were attached to a postsynaptic membrane (Fig. 5). MBP's (with or without synaptic vesicles) also contained amorphous ground material of smooth or rough surfaced endoplasmic reticulum, multivesicular bodies, neurosecretory coated vesicles and bundles of fibrillar material. The free mitochondria were

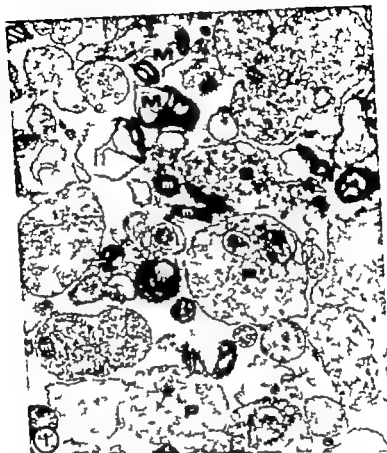


Fig. 1. P₂ fraction, hemisphere, glutaraldehyde fixation in pellet, no incubation. Several membrane-bound particles (MBP) are seen. Some of them contain spherical synaptic vesicles (arrow) and can be considered pinched off nerve endings (F). Side others (P) are devoid of synaptic vesicles. MBP have an associated substructure and also contain small mitochondria (m), tubules (thick arrow) and fibrils (double arrow). Other components are the free mitochondria (M) and microsomes (MTC) $\times 30,000$.

rupted, but were recognized because of their cristae and double membranes. Sometimes they were shrunken, with a dark appearance.

Compared to the adult (Hervonen *et al.* 1974) the main differences were: the absence of myelin, the small number of synaptosomes containing synaptic vesicles, and the large number devoid of synaptic vesicles. On the other hand, in the 1-day-old rat some myelin was found and the number of synaptosomes was greater (Hervonen *et al.* 1974).

Fraction A

This fraction consists mainly of unit membrane fragments (Fig. 5). They are usually curved and of very variable length. No myelin fragments were found. If myelination has begun at this stage, the myelin breaks down into single unit membranes during homogenization. MBPs in this fraction are fewer and smaller than in the following subfractions. This

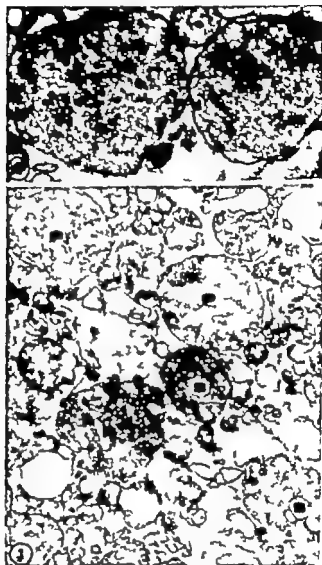


Fig. 2. P_1 -fraction, brain stem, glutaraldehyde-fixation in suspension, no incubation. Two MBP's of one (P) is devoid of synaptic vesicles, and the other fully mature synaptosome. The pre- and post-synaptic membrane thickenings and some cleft material between them can be seen. Note the small size of dense-cored vesicles (about 500 Å, arrow), and the larger size of third (open arrow) 37 900.

Fig. 3. P fraction, brain stem, triple-fixation, 5-OHDA incubation. The fine structure after triple-fixation resembles that after glutaraldehyde- OsO_4 -fixation (Fig. 1-2). Two (S) of the MBP's contain several synaptic vesicles, while others are devoid of them. The synaptic vesicles are small and granular. A coated cisterna is also observed (arrow), but not small granular vesicles. Several membrane particles can be seen (arrow). 21 400.

applies to MBP's with synaptic vesicles, *i.e.* synaptosomes (see Table I). The synaptosome cannot be distinguished morphologically in the different subfractions.

Fraction B

The number of membrane fragments is smaller than in fraction A, whereas the number and size of MBP's is larger. The MBP's usually contain only few synaptic vesicles (developing synaptosomes) but a few mature looking nerve endings can be found (Fig. 6). The

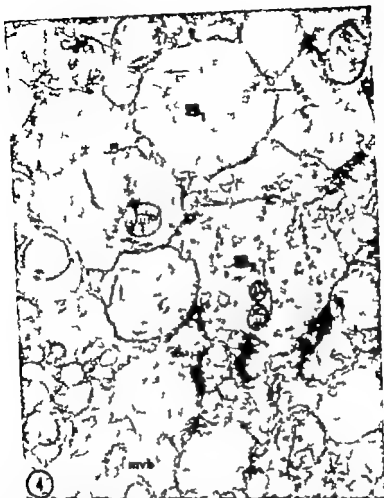


Fig. 4 P₂-fraction, heterophase, permanganate fraction on pellet, α -naphthyl-NA incubation. Most of the profiles are derived of synaptic vesicles or contain only a few of them (S₁, arrow). One nature looking exapoposome (S₂) with small granular vesicles, some large granular vesicles (tuxed arrow) and small ultracytoplasmic mitochondria (m) are seen. One MRP contains multivesicular body (mvb). 34 700

ner endings are packed with synaptic vesicles and display postsynaptic membrane thickening. The percentage of presynaptic terminals was calculated to be 1.8% (see Table 1).

Fraction C

This fraction together with fraction B, represents the main synaptosomal fraction in the fetal rat brain (see Table I). MBP's predominate in the fraction, which also contains some free mitochondria (Fig. 7). The MBP's are larger than in fraction B. They contain amorphous ground material and few organelles. They contain vesicles of the following types:

(1) *Small spherular vesicles (54%)* These are most common. They are spherical and between 200 and 600 Å in diameter (mean 420 Å, calculated from 500 vesicles). Usually there

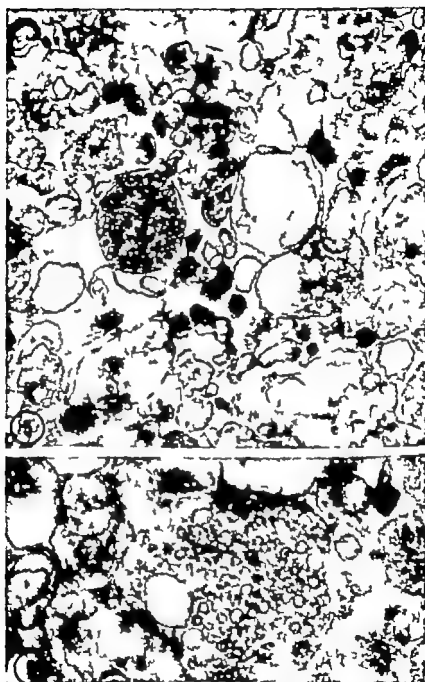


Fig. 5. A-fraction, whole brain, triple fixation, 5-OHDA incubation. One synaptosome (arrow) with SAV. Most of the fraction consists of membranes. 37 200.

Fig. 6. B-fraction, whole brain, triple fixation, 5-OHDA incubation. This synaptosome has the terminal of a mature nerve ending: It is packed with synaptic vesicles and displays symmetric postsynaptic membrane thickenings. Some vacuoles are seen in the synaptosome. 58 300.

are relatively few such vesicles per nerve ending, but the more mature nerve end is packed with these vesicles as in adult synaptosomes.

(2) *Dense core vesicles (DCV)* These are usually intermingled with SAV in samples. Most are between 500 and 800 Å in diameter (mean 620 Å calculated from 100

TABLE I Rat synaptosome fractions in 19-day-old fetus.

Brain region	Fraction	Total number of coated membrane-bound particles (MBP's)	MBP's without synaptic vesicles	MBP's with synaptic vesicles - pre-synaptic terminals	Percentage of pre-synaptic terminals
crustiform	F2	1 000	991	9	0.9
granular	F2	1 000	977	23	2.3
whole brain	A	1 000	993	7	0.7
	B	1 000	982	18	1.8
	C	1 000	968	31	3.2
	D	1 000	994	6	0.6
	E	1 000	995	5	0.5

and are thus smaller than the large granular vesicles (LGV) in adult nerve endings. They are also found alone in MBP's without other synaptic vesicles and may have functions different from neural transmission. Permanganate fixation does not demonstrate a dense core in these vesicles in the 19-day-old fetal brain.

(3) *Coated vesicles*. These are found both in MBP's without other organelles and in nerve endings containing the above types of synaptic vesicles. They are mostly 600-900 Å in diameter (mean 760 Å calculated from 100 vesicles).

(4) *Large agranular vesicles*. These vesicles, 600-2 000 Å in diameter are found among other synaptic vesicles or occasionally alone. They might be part of the smooth endoplasmic reticulum and/or represent developing or degenerating synaptic vesicles (Kanerva *et al* 1975).

Synaptosomes packed with so-called small granular vesicles (SGV) typical of monoaminergic nerve endings (Hokfelt *et al* 1970 Kanerva *et al* 1976, 1977) have not been observed in the fetal rat.

The attached postsynaptic membrane which is frequently seen in adult synaptosomes (Hervonen *et al* 1974) was observed, but not frequently. The adjacent synaptic membranes were either symmetrically thickened or usually the postsynaptic membrane was thicker. This is indicative of the mature appearance of synapses. The synaptic cleft measured 200 Å and contained some cleft material.

Fraction II

This fraction consists mainly of MBP's and free mitochondria. The MBP's are larger than in fraction C. They only infrequently contain synaptic vesicles, thus the number of synaptosomes or developing synaptosomes is small compared to fraction C. The large MBP's contain more organelles than MBP's in the lighter subfractions (such as mitochondria, rough or smooth endoplasmic reticulum, free ribosomes, fibril bundles, microtubules, vacuoles, granular and agranular vesicles). They might be derivatives of dendrites, perikarya or glial cells rather than pinched off axons. The free mitochondria are large and often disrupted.

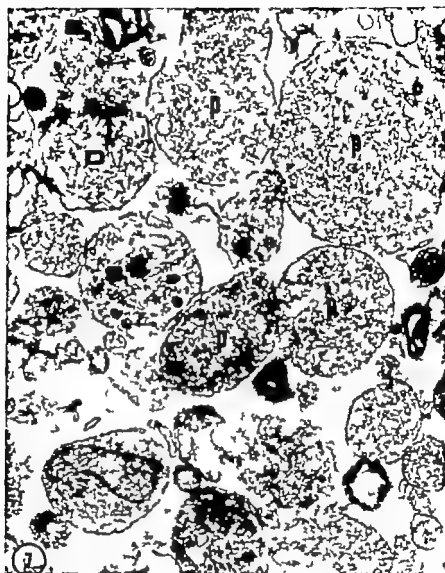


Fig. 7 C-fraction, whole brain, glutaraldehyde-fixation 1 suspension, no incubation. Several MBP's are seen. One (S_1) contains 3 dense-cored vesicles (DCV) (arrow), while another (S_2) with synaptic vesicles is connected to a dendrite (D) containing microtubules, microtubules and some ribosomes. The other MBP's (S) consist mainly of amorphous ground material. A coated vesicle is seen in one of them (thick open arrow).

Fraction E

The main constituents are large free mitochondria. MBP's of the same appearance as Fraction D are encountered and a few synaptosomes can be found.

Fine Structure after Different Fixatives

Compared to glutaraldehyde followed by osmication (Fig. 1-2, 7) the triple fixation method gave somewhat inferior preservation with more vacuoles in the MBP's (Fig. 3 5-6) and some structures were observed with this fixation. After permanganate (Fig. 4)

difficult to obtain good ultrathin sections. In the fetal rat synaptosomes, we were not able to demonstrate small granular vesicles (SGVs) characteristic of monoaminergic synaptosomes even after incubations with "false" transmitters (see below).

Regional Variations

The synaptosomes from the different brain areas, i.e. the brain stem, the hemispheres or the whole brain were identical in morphology. We counted the number of MBPs from the P₂ layer of the brain stem and the hemispheres (Table I). 2.8% of MBPs contained synaptic vesicles in the brain stem preparation vs. 0.9% in the hemisphere preparations. This seems to confirm our previous assumption that nerve endings in the brain stem develop earlier than those in the hemispheres (Hervonen *et al.* 1974).

Effects of Incubations

Contrary to our previous results in the 1-day-old rat (Kamara *et al.* 1976, 1977) small DCVs could not be demonstrated after incubation in either 5-OHDA or α -methyl-NA. This reflects the immaturity of the monoamine-storing vesicle pool at this stage of development. The appearance after incubation was largely unchanged irrespective of fixation method.

Discussion

The formation of synaptosomes is believed to result principally from differences in mechanical and physical properties between the nerve ending and the structures which surround it (Whittaker 1965). It seems generally accepted that by using the subfractionation techniques for nervous tissue, the synaptosomal fractions obtained are relatively pure in sheared off nerve endings (Whittaker 1963, 1972, Jones 1972, 1975). Other components in the adult synaptosomal fractions (fraction C with the method used in the present study) are few free mitochondria, some membrane fragments and few unidentified bodies. Lemkey-Johnston and Deldemirjian (1970), on the other hand, were of the opinion that synaptosome preparations are more heterogeneous, also containing large numbers of axonal segments and membranes of undetermined origin. However their subfractionation technique has been criticized (Jones 1972). During ontogenesis, the neurons (and the glial cells) might have different mechanical and physical properties. During development, neurons differ from those of the adult (Tennysen 1970 a, b). Prominent structures during development are the growth cones (Kawana *et al.* 1971, Vaughn *et al.* 1974). Several electron microscopic studies on the growth cone have appeared during the last years (Bodian 1966, Bodian *et al.* 1968, Del Cerro and Souder 1968, Tennysen 1970 b, Kawana *et al.* 1971, Vaughn *et al.* 1974). The growth cones consist of a bulging cytoplasm from which extremely fine filopodia protrude. The main structural characteristics of the cone-shaped area are the smooth endoplasmic reticulum complexes. These are organized as tubules, sacs and vesicles. Other components are microtubules, filaments, mitochondria, coated invaginations of plasma membranes, coated vesicles, ribosomes and synaptic vesicles. The similar arrangement of endoplasmic reticulum in the

growth cones and in the synaptosomes allows us to suggest that some of the most bounded particles observed during ontogenesis are derived from growth cones. In the way that pinched off nerve endings are formed in adult animals, pinched off growth cones might be formed during subfractionation of immature nervous tissue. Since growing dendrites also display growth cones (Kawana *et al* 1971, Vaughn *et al* 1974) it might be that a larger proportion of membrane bound particles are derived from dendrites during ontogenesis than in adults. Kawana *et al* (1971) were unable to differentiate with certainty axonal and dendritic growth cones *in vitro*. Thus, probably they cannot be differentiated after the subfractionation procedure. In summary it seems that after subfractionation of immature nervous tissue, the synaptosomal fractions contain pinched off growth cones from axons and dendrites and perhaps from glial cells, in addition to the normal constituents of the synaptosomal fractions (synaptosomes, mitochondria, some membrane fragments and a few unidentified bodies).

During the last decade several investigations have dealt with the fine structure of synaptogenesis in several animal species (Tennyson 1970 a). In the rat postnatal synaptogenesis has been investigated in many brain areas including the neocortex, the lateral geniculate nucleus, the olfactory bulb, the parietal cortex, the cerebellar cortex, the hippocampus, the superior colliculus, the dentate gyrus, the corpus striatum and the lateral vestibular nucleus (Tennyson 1970 a, Hervonen *et al* 1974). Some reports on fetal synaptogenesis have appeared (Kobayashi *et al* 1968, Dalkoku *et al* 1971, Del Cerro and Snider 1972, May-Bascoe 1973, Vaughn and Gricahaber 1973, König *et al* 1975). Judging from the currently available literature, it seems reasonable to assume that while mature synapses can be formed during prenatal development in the rat the period of rapid increase in number of synapses in most parts of the brain is during the first three postnatal weeks (e.g., Aghajanian and Bloom 1967, Woodward *et al* 1971). This interval between the time at which synapses first formed and the time of rapid increase in number also applies to mammals with a mature nervous system at birth. For example in the cat visual system in which a few dendritic synapses are present as early as three weeks before birth the rapid increase of synapses occurs 8-37 days after birth (Cragg 1974). The present finding of developing synapses during the prenatal development fits well with the above concept and holds promise for future work on the immature synapse using the subfractionation technique.

The synaptic vesicles are mainly small agranular vesicles (SAV) and large granular vesicles (LGV). In the fetal synaptosomes, SAV were of the same size (mean 420 Å) as reported in adults (Whittaker 1972). This is in agreement with a study on the median eminence of the rat where SAV did not appear to change in size during pre- or postnatal development (Kobayashi *et al* 1968, Dalkoku *et al* 1971). LGV on the other hand, were reported to increase in size, most being 600-800 Å in diameter in the 18-day-old fetus, later increasing to 1000 Å at which size they remained during postnatal development. In the present study the size of LGV (mean 620 Å) is smaller than reported for adults (Whittaker 1972). The LGV were solitary among large numbers of SAV in the synaptosomes and can hardly be called so-called small granular vesicles (SGV) containing monoamines (Hökfelt 1973). Pinner *et al* (1969) have tentatively proposed that there is a close relationship between dense-cored vesicles and dense projections. We have not been able to confirm this.

in this or our other works on the ontogenesis of nerve endings (Hervonen *et al.* 1974a, *et al.* 1974b). Regarding the genesis of synaptic vesicles, it seems that their origin is almost every component of the neuron has been proposed: mitochondria, macro-ribosomes, cell membrane, complex vesicles, large granular vesicles, the Golgi apparatus, and smooth endoplasmic reticulum (SR) (for ref. see Hökfelt 1973, Kanerva *et al.* 1974a). The co-existence of synaptic vesicles and smooth endoplasmic reticulum in the membrane-bound particles (MBP's) is of interest. These two elements also share an affinity for the zinc-iodine-osmium (ZIO) stain (Kawana *et al.* 1971) and it is possible that the small agranular vesicles are SR derivatives. One step during this formation might be the large agranular vesicles (Kanerva *et al.* 1975a). The dense core of the SGV on the other hand has been observed to form within SR tubules (Hervonen and Kanerva 1973, Hökfelt 1973, Kanerva *et al.* 1976).

The best results in demonstrating monoamines with electron microscopy have been obtained by (1) permanganate fixation (Richardson 1966, Hökfelt 1973), (2) glutaraldehyde fixation with post-osmication after loading the nerve endings with "false transmitters" such as 5-OHDA, α -methyl-NA or 6-OHDA (Tranzer 1972, 1973, Hökfelt 1973) or (3) a modified method based on the chromaffin reaction (Wood and Barnett 1964) introduced by Tranzer and co-workers (Tranzer and Snipes 1968, Tranzer 1972, 1973, Tranzer and Richards 1976). It is claimed that this triple-fixation method (aldehydes, chromates and osmium tetroxide) (Tranzer 1972, 1973) permits the demonstration of both the so-called granular pool and the non-granular pool of monoamines. In the present paper we have used the above fixation procedures but were not able to demonstrate monoamine-structures in fetal rat synaptosomes. It has been suggested that the tubular reticulum which is believed to store the non-granular pool of monoamines (Erlink 1972, Tranzer 1972, 1973) is labile and the homogenization procedure could thus liberate their amine content during the processing of synaptosomes. We have previously demonstrated small granular vesicles in the 1-day-old rat although their number was small (Kanerva *et al.* 1976, 1977). Varicose nerve terminals have been demonstrated in the fetal rat brain by the formaldehyde-induced fluorescence (FIF) technique (Singer and Olson 1973). Since SGV were not found in the present study and monoamines can be demonstrated in the nerve endings with the FIF method in the fetal rat, we conclude that the granular pool of catecholamines is poorly developed or less stable than in adults and/or the cytochemical techniques used are defective in demonstrating the SGV in the central nervous system during this early stage of development.

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By

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Abstract

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The effect of moderate hypoxia (50.5 kPa air) and severe hypoxia (40.8 kPa air) *in vivo* on liver and heart triacylglycerol concentration and mitochondrial respiration rates was studied. Liver triacylglycerol concentrations increased in severe hypoxia from 7.3 $\mu\text{mol/g}$ wet weight to 23.3 $\mu\text{mol/g}$ wet weight over 7 days. At the period of seven days in severe hypoxia the palmitate, octanoate and palmitoylcarnitine oxidation in mitochondrial suspensions were significantly reduced when the citric acid cycle was operative. decrease in the fatty acid fatty acyl-CoA: carnitine derivative oxidation was observed when only β oxidation system was studied. Mitochondria isolated from the heart and liver after seven days in severe hypoxia showed reduced respiratory control ratios, the decrease being from the normal 4.9 to 1.9 in liver mitochondria using succinate as substrate. The reduction in respiratory control was mainly due to lowered State 3 respiration rates. Some reduction in the ratio was also observed in the fasting control from 5.8 to 3.4 with succinate. The respiratory control ratio could be partially normalized by the addition of albumin to the isolation medium for the liver mitochondria after severe hypoxia. Under these conditions however the State 4 respiration of the mitochondria from the hypoxic animals was higher than that for controls.

Key words. Heart, liver, hypoxia, triglycerides, fatty acid oxidation

The role of oxygen in the induction of the biogenesis of mitochondria and the synthesis of cytochromes in the lower eucaryotes is well established (Biggs and Linnane 1963). The view also emerges that oxygen availability may also be an important regulator of the synthesis of some of the components of mitochondria in mammals, both in cell cultures (Pious 1970) and *in vivo* (Kinnula 1976). There is also evidence that the postnatal increase in the amount of mitochondria may be accounted for by the increase in oxygen concentration (Kinnula 1971, Kinnula and Hassinen 1977).

There is some evidence that the capacity of the mitochondrial fatty acid oxidizing system is a positive correlation with the cytochrome content of the mitochondria, at least in stoichondria from hyperthyroid animals (Kadenbach 1966, Diamant *et al* 1972). The present study was therefore undertaken to test the possible inducing effects of oxygen on a special mitochondrial aerobic pathway the β oxidation of fatty acids.

This demonstrated that the capacity for fatty acid oxidation is impaired in hypoxia. This is probably due to a decrease not in the activity of the fatty acid oxidizing enzymes but in that of the mitochondrial respiratory chain. An accumulation of triglycerides occurs in the liver during hypoxia, probably due to the decreased fatty acid oxidation.

Material and Methods

Reagents Standard reagents are obtained mainly from E. Merck AG, Darmstadt, Germany. Fatty acids, amino acids, derivatives, alcohols and ADP were purchased from Sigma Chemical Co. St. Louis, Mo. U.S.A., and carbonyl-*l*-tryptophan-*p*-nitrofluorobenzyl-phenylhydrazones (CL-CCP) from Boehringer GmbH, Mannheim, Germany. Specific protein alkaline was obtained from Nov. Industri A/S, Bagsvaerd, Denmark.

Animals and in vivo experiments Adult Wistar rats of both sexes (age 3-4 months) were used. The periods consisted of one day or seven days in hypobaric pressure chamber at atmospheric pressure of 50.5 kPa or 40.5 kPa in the one-day experiments only after as suitable for the rats, but during the longer hypobaric periods they had access to food (standard rodent chow obtained from Jankrup Ltd. Jankrup, Finland) and water. Seven rats out of ten, when exposed to hypoxia, the effect of food restriction, as tested on one group of control animals by limiting food intake. Rats on the restricted diet are given food of 1 kJ/day (Zapfel 1967), during which time they consumed 5-10 g, compared with normal consumption of 20-25 g/day. With this arrangement the weight loss in the fasting controls is the same as in the rats undergoing severe hypoxia.

Isolation Liver mitochondria were isolated by conventional Schneider procedure, the isolation medium consisting of 0.25 M sucrose, 1 mM EDTA and 5 mM Tris-chloride (pH 7.4). The mitochondrial fraction was washed twice in some of the cases albumin was added to the isolation medium to final concentration of 2 (Martin and Dounce 1971). In this case one washing was included in the isolation. Heart mitochondria were isolated as previously described (Kuznetsov 1976), essentially according to Tyler and Goetts (1967). The isolation medium consisted of 0.225 M mannitol, 0.075 M sucrose and 0.05 mM EDTA (pH 7.4).

Usually *Bacillus subtilis* alkaline (EC 3.4.4.16.) was used during the isolation, though it is omitted, as indicated, in some experiments. Heart and liver triglycerides were determined by the method of Carlson (1963). The rate of oxidation of fatty acids is determined polarographically (Yellow Springs oxygen electrode 3331), essentially according to Garland (1968). Usually the mitochondrial suspension was added to an as-saturated medium consisting of 80 mM KCl, 20 mM Tris-chloride, pH 7.4, 5 mM malonate, 5 mM malate, 3 mM phosphate, 1 mM EDTA and 2 mM ADP. Malonate was omitted from the oxidation experiments on the heart mitochondria, and malonate or malate from those on the liver mitochondria in some cases. The concentration of palmitoyl-CoA was 3.7 μ M (Garland 1968). The concentration of palmitate and octanoate, less present, was 0.7 mM (Pande 1971, Hansen and Kishner 1974). The palmitoyl-CoA concentration was 3 μ M, and carnitine concentration 1.5 mM (Pande 1973). 1 μ M of succinate, 2-oxoglutarate or glutamate malate, the buffer consisted of 0.225 M sucrose, 10 mM phosphate (pH 7.4), 5 mM $MgCl_2$ and 20 mM KCl. Further additions were 5 mM substrates (State 4) and 0.25 mM ADP (State 3). All the oxidations were carried out at 22°C. Liver mitochondrial FAD and FMN concentrations are determined fluorimetrically and non-fluorescent DAPI spectrophotometrically according to King *et al* (1962). Protein determinations are carried out according to Lowry *et al* (1951). The Student's *t*-test was used for the statistical analysis, and *P* values of 0.05 were considered to indicate statistical significance.

In further experiments (to be reported elsewhere) pair-feeding of the experimental animals was used. The food intake data showed that the method of food restriction used in the present communication reproduces consumption of the hypoxic animals quite accurately.

Results and Discussion

An atmospheric pressure of 50.5 kPa represents moderate hypoxia to which adaptation not difficult, as is observed in previous studies (Kinnula 1975, 1976), whereas hypoxia the level of 40.8 kPa proved to be so severe that the animals were at the limits of their survival capacity and 9.5% died. The mechanism of death was not investigated, but all deaths occurred during the first 3 days. At both pressures a clear hypertrophy of the heart was seen, which was more prominent at 40.8 kPa, about 30%, when expressed as g/100 b wt. Only in severe hypoxia did any decrease in liver occur, about 20% over 7 days. The corresponding weight decrease in the controls on the restricted diet was 22%, however.

Subtilopeptidase A was used here for the isolation of heart mitochondria. Since evidence has been presented of a susceptibility of ATP dependent long-chain fatty acid activating enzyme to the action of Subtilopeptidase A ("Nagase") in liver and heart (Van Tol-Hülsman 1970, Pande and Blanchard 1970), its effect on the isolation of heart mitochondria was tested, revealing no deleterious effects. Indeed, it had certain advantages, viz. an increase in the yield of mitochondria, which are also more stable, as evaluated from respiratory control ratios (Table I).

Uncoupling of mitochondria isolated from fat-containing tissues can be prevented by including albumin in the isolation medium (Martin and Denton 1971), and thus, as an accumulation of triglycerides in the liver was observed in this study (see below), albumin was also used in some of the expts. the final concentration in the isolation medium of liver mitochondria being 2%.

Fatty acid oxidizing capacity From Fig. 1 and 2 it can be observed that under conditions allowing complete oxidation of fatty acids to CO₂ and water the rate of oxidation in hypoxia declined systematically, this effect being most prominent after seven days at 40.8 kPa. The effect of albumin treatment on fatty acid oxidation under non-ketogenic conditions was tested. The direction of the changes was the same for all the fatty acids studied, i.e. palmitate.

TABLE I Effect of *B. subtilis* alkalase on respiratory rates and respiratory control ratios using succinate as a substrate and the oxidation of palmitate, palmitate + carnitine and palmitoylcarnitine by liver mitochondria. Values are means \pm S.D. from the number of expts. indicated in parentheses. Oxygen consumption is expressed as μ mol/min/g protein (22°C). The mitochondrial suspension was added in an air-saturated medium consisting of 80 mM KCl, 20 mM Tris-chloride pH 7.5, 5 mM malate, 5 mM phosphate, 1 mM EDTA and 2 mM ADP. Palmitate was added at the beginning of the expt. and carnitine 5 min later. Oxidation of palmitoylcarnitine was measured in separate expts.

	<i>B. subtilis</i> alkalase	Alkalase omitted
Succinate, State 4	39.6 \pm 5.8 (6)	31.8 \pm 7.1 (6)
Succinate, State 3	134.2 \pm 17.8 (6)	39.7 \pm 9.3 ^a (6)
Respiratory control ratio	3.5 \pm 0.4 (6)	1.9 \pm 0.7 ^a (6)
Palmitate, State 3	47.1 \pm 6.9 (5)	35.2 \pm 7.3 (5)
Palmitate + carnitine, State 3	67.4 \pm 10.2 (5)	54.0 \pm 9.9 (5)
Palmitoylcarnitine, State 3	116.1 \pm 12.4 (5)	106.0 \pm 13.9 (5)

Fig. 1 Oxidation of palmitate, palmitoylcarnitine and octanoate by rat liver mitochondria after different hypoxic periods. Control values represent 100%. Δ — Δ , \circ — \circ palmitate and \square — \square , \triangle — \triangle palmitoylcarnitine. Left: moderate hypoxia (5 kPa). Right: severe hypoxia (40 kPa). Medium: See Table II, no albumin in the isolation medium.

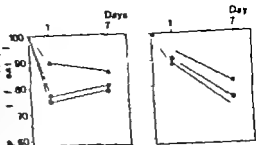
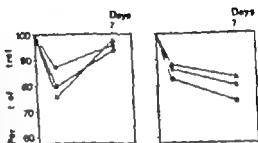


Fig. 2 Oxidation of palmitate, palmitoylcarnitine and octanoate by heart mitochondria after different hypoxic periods. Control values represent 100%. Δ — Δ , \circ — \circ palmitate and \square — \square , \triangle — \triangle palmitoylcarnitine. Left: moderate hypoxia (5 kPa). Right: severe hypoxia (40 kPa). Medium: See Table II. Bovine albumin was used during the isolation.



palmitoylcarnitine and octanoate. The initial values, corresponding to the 100% values in Fig. 1 and 2, are presented in Table II.

The fatty acid β oxidation capacity during inhibition of the citric acid cycle is depicted in Table III. No significant differences can be observed between the control and hypoxic groups in the palmitoylcarnitine or palmitoyl-CoA oxidation experiments, and only a slight decrease can be noticed in the palmitate oxidation capacity in the hypoxic mitochondria. In these experiments albumin was added to the isolation medium and malonate but not malate was used in the oxygen consumption experiments. The citric acid cycle was inoperative. The oxygen consumption was only due to β oxidation under ketogenic conditions. Since the palmitoylcarnitine and palmitoyl-CoA oxidation capacities were normal, neither the formation of the activated fattyacyl-CoA nor any of the oxidation steps of the palmitoylcarnitine could have been inhibited. These results differ from those experiments in which the citric acid cycle is not inhibited (Fig. 1 and 2). With complete oxidation to CO_2 a significant decrease was observed in the fatty acid oxidizing capacity possible due to a decline in the capacity of the mitochondrial respiratory chain to oxidize the reducing equivalents generated by the citric acid cycle, the latter being apparent only under conditions of high rates of cycle turnover.

Mitochondrial flavins. Mitochondria contain at least three distinct flavin entities, FAD and FMN in the acid-extractable fraction and non-fluorescent flavin in the acid non-extractable fraction. All these flavins were determined from liver mitochondria after severe seven-day hypoxia. The FAD concentration was $0.43 \pm 0.02 \mu\text{mol/g}$ mitochondrial protein in the control group and $0.39 \pm 0.06 \mu\text{mol/g}$ mitochondrial protein in the hypoxic group, the respective FMN concentrations being 0.09 ± 0.01 and $0.10 \pm 0.02 \mu\text{mol/g}$ protein and those of non-fluorescent flavins (like succinate dehydrogenase) $0.15 \pm 0.02 \mu\text{mol/g}$ protein and $0.16 \pm 0.02 \mu\text{mol/g}$ protein. All the results are means from 4 or 5 determinations, and

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Fatty acid oxidizing capacity. From Fig. 1 and 2 it can be observed that under conditions allowing complete oxidation of fatty acids to CO₂ and water the rate of oxidation in hypoxia declined systematically, this effect being most prominent after seven days at 40.8 kPa. The effect of albumin treatment on fatty acid oxidation under non-ketogenic conditions was not tested. The direction of the changes was the same for all the fatty acids studied, i.e. palmitate.

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	<i>B. subtilis</i> alkalase	Alkalase omitted
Succinate, State 4	39.6 \pm 5.8 (6)	31.8 \pm 7.2 (6)
Succinate, State 3	134.2 \pm 17.8 (6)	59.7 \pm 9.8* (6)
Respiratory control ratio	3.5 \pm 0.4 (6)	1.9 \pm 0.2* (6)
Malate, State 3	47.1 \pm 6.9 (5)	35.2 \pm 7.3 (5)
Palmitate + carnitine, State 3	67.4 \pm 10.2 (5)	34.0 \pm 9.9 (5)
Palmitoylcarnitine, State 3	116.1 \pm 12.4 (5)	106.0 \pm 15.9 (5)

TABLE III. Oxidation of palmitoylcarnitine, palmitate, carnitine and palmitoyl-CoA + carnitine after severe hypoxia at 40 kPa for 7 days in albumin-treated rat liver mitochondria under ketogenic conditions. The data are presented as $\mu\text{moles}/\text{min}/\text{g}$ protein. Values are means \pm S.D. from the number of experiments indicated in parentheses. The mitochondrial suspension was added to an air-saturated medium consisting of 80 mM KCl, 20 mM Tris-Chloride pH 7.4, 5 mM phosphate, 1 mM EDTA, 5 mM malonate and 2 mM ADP. Palmitate was added at the beginning of the experiment and the rate of oxygen consumption recorded, after which carnitine was added (2 min later) and the rate of oxygen consumption measured again. Oxidation of palmitoylcarnitine as measured in separate experiments.

	Control	Hypoxic
Palmitoylcarnitine	6.40 ± 1.44 (8)	6.63 ± 1.00 (8)
Palmitate	3.30 ± 0.75 (8)	3.01 ± 0.72 (8)
carnitine	4.80 ± 0.95 (6)	4.92 ± 0.70 (6)
Palmitoyl-CoA	3.34 ± 0.82 (6)	3.69 ± 0.60 (6)
carnitine	4.26 ± 0.76 (6)	4.42 ± 0.74 (6)

adaptation is usually impossible, decreased activities are obtained (Ziegler 1967, Kim and Han 1969, Gold and Costello 1974).

The effect of food restriction on mitochondrial respiration was studied as food consumption has been observed to decline in hypoxia. The weight loss of the controls on the restricted diets and the animals undergoing severe hypoxia was the same, about 20%. State 3 respiration rates and respiratory control ratios of liver mitochondria isolated from the animals on food restriction decreased from the normal 5.8 to 3.4 with succinate, from 6.6 to 3.6 with 2-oxoglutarate and from 5.8 to 3.8 with glutamate + malate (all values are means from 3 determinations). State 4 respiration remained unchanged. In this respect the hypoxic and fasting groups show quite similar results, though the decrease in the respiratory control ratios was smaller after fasting.

TABLE IV. Effect of hypoxia on the oxidation of succinate by liver mitochondria and the respiratory control ratios (RC) using succinate or glutamate + malate as substrate (no albumin in the isolation medium). The data are presented as $\mu\text{moles}/\text{min}/\text{g}$ protein. Values for succinate are means \pm S.D. from 6-8 determinations. Respiratory control ratios for glutamate + malate are means from 3 determinations.

			Succinate		Glutamate + malate	
			State 4	State 3	RC	RC
Control						
1 day	1		10.6 ± 2.8	52.9 ± 15.2	5.2 ± 0.5	6.3 ± 0.9
7 days	2		10.5 ± 0.7	46.5 ± 11.6	4.4 ± 0.5	5.4 ± 1.1
1 day	3		10.7 ± 2.2	51.7 ± 14.7	4.9 ± 0.6	5.8 ± 0.8
7 days	4		10.1 ± 1.6	45.5 ± 9.9	4.9 ± 0.5	5.3 ± 0.6
30 kPa						
1 day	1		12.0 ± 2.0	55.2 ± 14.8	5.1 ± 0.5	5.3 ± 0.6
7 days	2		11.9 ± 2.0	58.5 ± 10.8	5.0 ± 0.8	5.7 ± 0.7
40 kPa						
1 day	3		11.7 ± 1.9	45.9 ± 4.7	3.7 ± 0.7^a	3.9 ± 0.3
7 days	4		12.0 ± 1.1	23.8 ± 7.1	1.9 ± 0.9^b	3.1 ± 0.2

TABLE II Oxidation of palmitate, palmitoylcarnitine and octanoate by liver and heart mitochondria of control rats. Values are means \pm S.D. from the number of expts. indicated in parentheses. The data are presented as μ atoms/min/g protein (22°C). Initially the mitochondrial suspension was added to an air-saturated medium consisting of 80 mM KCl, 20 mM Tris-chloride pH 7.4, 5 mM malate, 5 mM phosphate, 1 mM EDTA and 2 mM ADP. Palmitoylcarnitine was added 1 s after the addition of palmitate.

	Heart	Liver
Palmitate	43.1 \pm 9.5 (18)	11.5 \pm 3.6 (18)
Palmitoylcarnitine	73.2 \pm 9.0 (18)	29.3 \pm 4.0 (18)
Octanoate	34.5 \pm 1.9 (18)	13.9 \pm 2.0 (18)

hypoxia induced differences were not statistically significant. These results are in full agreement with the results of unchanged fatty acid β oxidation stated above. Any large increase or decrease in the amounts of fatty acid oxidizing enzymes which include two flavoproteins (the acyl-CoA dehydrogenase and the electron transferring flavoprotein) would be probably reflected in an increase or decrease in the total flavin concentration of the tissues, as observed previously (Hassinen and K  h  nen 1974). In intact tissues and mitochondrial suspensions, fatty acid oxidation results in extremely large changes in the fluorescence and absorbance of flavins (Hassinen and Hiltunen 1975; Garland *et al.* 1967), which emphasize the role of the flavins.

Respiratory control ratio. No changes in State 4 or State 3 (Chance and Williams 1955) oxidation rates of glutamate + malate and succinate were induced by moderate hypoxia, nor was any significant change in State 4 respiration observed with either substrate in severe hypoxia. In severe hypoxia, a significant decrease in respiration of liver mitochondria in State 3 was observed (Table IV). In these expts. albumin was not used in the isolation medium. The results for the heart mitochondria are shown in Table V. In this tissue also, State 3 respiration rate was significantly reduced in severe hypoxia.

The reduced respiratory control ratios were not due to uncoupling of the mitochondria, since State 4 respiration rates remained almost unchanged. It is more likely that the decrease in State 3 respiration results from inactivation of succinate dehydrogenase, or adenine nucleotide translocase or the reduced capacity of the respiratory chain proper. This interpretation is also supported by the finding that this difference can only be demonstrated under conditions of high turnover in the respiratory chain. The decrease in the respiration rate with succinate in severe hypoxia after seven days was statistically significant, whereas in moderate hypoxia a slight increase in State 3 respiration occurred ($+26\%$) indicating increased succinate dehydrogenase activity (Table IV). There is still great variation in the reports on the effect of hypoxia on succinate dehydrogenase activity. One reason is undoubtedly the diversity of the assay conditions in different laboratories and the activation-inactivation phenomenon of succinate dehydrogenase *in vitro*. It is also known that the activity of succinate dehydrogenase undergoes short-term modulation independently of enzyme synthesis (Aithal and Ramasarma 1969). In most studies increased succinate dehydrogenase activities have been observed in moderate hypoxia (Aithal and Ramasarma 1969; Susheela and Ramasarma 1973a; 1973b; Sherzer and Cascarano 1972; Kinnula 1975), whereas in severe hypoxia they are

TABLE III. Oxidation of palmitoylcarnitine, palmitate, carnitine and palmitoyl-CoA + carnitine after severe hypoxia at 40 kPa for 7 days in albinized-treated rat liver mitochondria under ketogenic conditions. The data are presented as means \pm S.D. Values are means \pm S.D. from the number of experiments indicated in parentheses. The mitochondrial suspension is added to an air-saturated medium containing 80 mM KCl, 20 mM Tris-Chloride pH 7.4, 5 mM phosphate, 1 mM EDTA, 5 mM malonate and 2 mM ADP. Palmitate is added at the beginning of the experiment and the rate of oxygen consumption recorded, after which carnitine is added (1 min later) and the rate of oxygen consumption measured again. Oxidation of palmitoylcarnitine is measured in separate experiments.

	Control	Hypoxic
palmitoylcarnitine	6.40 \pm 1.64 (8)	6.43 \pm 1.03 (8)
palmitate	3.30 \pm 0.73 (8)	3.01 \pm 0.77 (8)
carnitine	4.80 \pm 0.93 (6)	4.92 \pm 0.70 (6)
palmitoyl-CoA	3.36 \pm 0.82 (6)	3.99 \pm 0.40 (6)
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adaptation is usually impossible, decreased activities are obtained (Ziegler 1967, Kim and Han 1969, Gold and Costello 1974).

The effect of food restriction on mitochondrial respiration was studied as food consumption has been observed to decline in hypoxia. The weight loss of the controls on the restricted diets and the animals undergoing severe hypoxia was the same, about 20%. State 3 respiration rates and respiratory control ratios of liver mitochondria isolated from the animals on food restriction decreased from the normal 5.8 to 3.4 with succinate, from 6.6 to 3.6 with 2-oxoglutarate and from 5.8 to 3.8 with glutamate + malate (values are means from 3 determinations). State 4 respiration remained unchanged. In this respect the hypoxic and fasting groups show quite similar results, though the decrease in the respiratory control ratios was smaller after fasting.

TABLE IV. Effect of hypoxia on the oxidation of succinate by liver mitochondria and the respiratory control ratios (RC) using succinate or glutamate + malate as substrate (no ethanol in the medium). The data are presented as means \pm S.D. from 6-8 determinations. Respiratory control ratios for glutamate + malate are means from 3 determinations.

		Succinate			Glutamate + malate
		State 4	State 3	RC	RC
Control					
1 day	1	10.6 \pm 2.8	32.9 \pm 13.2	5.2 \pm 0.5	6.3 \pm 0.9
7 days	2	10.5 \pm 0.7	46.5 \pm 11.6	4.4 \pm 0.5	5.4 \pm 1.1
1 day	3	10.7 \pm 2.2	31.7 \pm 14.7	4.9 \pm 0.6	5.6 \pm 0.8
7 days	4	10.1 \pm 1.6	43.5 \pm 9.9	4.9 \pm 0.5	5.3 \pm 0.6
30.5 kPa					
1 day	1	12.0 \pm 2.0	33.2 \pm 14.8	5.1 \pm 0.5	5.5 \pm 0.6
7 days	2	11.9 \pm 2.0	36.5 \pm 10.8	5.0 \pm 0.8	5.7 \pm 0.7
40.8 kPa					
1 day	3	11.7 \pm 1.9	45.9 \pm 4.7	3.7 \pm 0.7 ^a	3.9 \pm 0.3
7 days	4	12.0 \pm 1.1	23.8 \pm 7.1 ^b	1.9 \pm 0.9 ^b	3.1 \pm 0.2

^a $p < 0.05$, ^b $p < 0.001$

TABLE V Effect of hypoxia on the oxidation of succinate by heart mitochondria and the respiratory control ratios (RC) using succinate or glutamate+malate as a substrate. The data are given as $\mu\text{atoms/min/g protein}$. Values for succinate are means \pm S.D. from 8-11 determinations; values for glutamate+malate means \pm S.D. from 3-4 determinations.

		Succinate			Glutamate + malate
		State 4	State 3	RC	RC
Control					
1 day	1	33.8 ± 6.2	127.4 ± 18.1	4.0 ± 0.4	4.0 ± 0.5
7 days	2	26.6 ± 5.8	112.5 ± 34.9	3.9 ± 0.5	3.8 ± 0.4
1 day	3	31.8 ± 10.7	115.3 ± 12.4	4.0 ± 0.5	3.9 ± 0.4
7 days	4	25.8 ± 5.1	105.3 ± 10.2	3.7 ± 0.5	4.0 ± 0.4
50.5 kPa					
1 day	1	34.7 ± 5.8	123.1 ± 13.9	3.7 ± 0.4	3.9 ± 0.3
7 days	2	26.2 ± 11.1	146.9 ± 15.3	5.1 ± 0.2	4.0 ± 0.2
40.8 kPa					
1 day	3	26.0 ± 1.5	101.8 ± 18.2	3.9 ± 0.9	3.4 ± 0.5
7 days	4	28.7 ± 6.3	72.8 ± 10.3^a	2.9 ± 0.6^b	3.4 ± 0.1

$p < 0.01$ $^b p < 0.05$

When the liver mitochondria were prepared in the presence of albumin a slight increase in State 4 respiration was observed (Table VI). This indicates a small degree of uncoupling such as is not apparent in mitochondria prepared without albumin and containing exogenous fatty acids. The expts. on the effects of the uncoupler Cl-CCP on the mitochondria proved unreliable in the presence of albumin because of the variable binding of the uncoupler to the traces of albumin present (data not shown).

There is evidence that fatty acids induce swelling in mitochondria and inhibit some mitochondrial enzyme activities, e.g. citrate synthase (Tubbs 1963; Wieland and Weiss 1964). The albumin requirement for the isolation of mitochondria of good quality from the liver of hypoxic animals indicates that the properties of these mitochondria are partly due to increased free fatty acid concentration in the liver cells. In experiments on hypoxic mitochondria isolated without albumin, State 3 respiration was markedly lower without any change in State 4 respiration compared with normoxic conditions. This means that the effect is not simply due to an uncoupling, for if this were the case the decline in the respiratory control ratios in the hypoxic mitochondria would result in an increase in State 4 respiration, not a decrease in State 3 respiration. Reduced respiratory control ratios and slower State 3 respiration rates have also been found when mitochondria are isolated without albumin from perfused ischemic liver (Bolme *et al.* 1970). In that case the decline in the respiratory control ratios parallels the rise in the free fatty acids and decrease in cardiolipin in the mitochondria.

Triglycerides. A significant accumulation of liver triglycerides occurred during the severe 7-day hypoxic period (Table VII) though moderate hypoxia does not cause any deposit of triglycerides. The origin of the fatty acids in hypoxia-induced triglyceride accumulation is uncertain but lipolysis from the adipose tissue may partly contribute to it. Serum free fatty acid levels were unchanged after seven days in severe hypoxia (Table VII), but their concentration had increased by 22% after one day under the same conditions (data not shown).

TABLE VI Effect of hypoxia for 7 days at 40.8 kPa on the oxidation of succinate, 2-oxoglutarate and glutamate substrate by liver mitochondria with albumin added to the isolation medium. The data are presented as $\mu\text{mol/min/mg}$ protein. Values are means \pm S.D. from the number of experiments indicated in parentheses.

	State 4	State 3	RC
Succinate			
Control	7.6 ± 1.1 (10)	41.2 ± 7.1 (10)	5.5 ± 1.2 (10)
Hypoxic	11.0 ± 3.4^a (10)	42.6 ± 7.4 (10)	4.2 ± 0.9^b (10)
2-oxoglutarate			
Control	2.6 ± 0.7 (5)	14.1 ± 5.8 (5)	3.2 ± 1.1 (5)
Hypoxic	2.9 ± 0.8 (5)	12.5 ± 2.6 (5)	4.7 ± 2.2 (5)
Glutamate oxidase			
Control	3.4 ± 1.1 (5)	19.4 ± 3.8 (5)	6.6 ± 2.2 (5)
Hypoxic	3.0 ± 1.4 (5)	17.2 ± 4.1 (5)	5.9 ± 2.1 (5)

p 0.002 p 0.001

reduced fatty acid oxidizing capacity however probably offers a more plausible explanation of the development of the hepatic steatosis. In this respect the mechanism may be similar to the development of ethanol-induced fatty liver which seems to be due mainly to an inhibition of hepatic fatty acid oxidation with a simultaneous activation of the fatty acid esterification pathways (Blomstrand and Kager 1974). It has been shown that even moderate hypoxia increases sympathoadrenal activity (Euler and Hellner 1952, Surks *et al.* 1967). Hypoglycemia has also been reported in hypoxia (Timiras *et al.* 1958) and prolonged hypoxia is known to result in a depletion of hepatic glycogen (Timiras *et al.* 1958, Girgis and Gold 1971). These are changes which are likely to exercise an adverse effect on lipogenesis in the liver. It has also been reported both with perfused livers (Salmon *et al.* 1974) and *in vivo* (Savolainen *et al.* 1977) that situations of carbohydrate depletion coincide with diminished fatty acid synthesis. Lactate can in perfused liver control lipogenesis by substrate limitation

TABLE VII Effect of 7-day hypoxia and food restriction (pellets for 1h/day) on tissue triglyceride concentrations and serum free fatty acid levels. Concentrations are expressed as $\mu\text{mol/g}$ wet weight for triglycerides and $\mu\text{mol/ml}$ for serum free fatty acids. Values are means \pm S.D. from the number of experiments indicated in parentheses.

	Triglycerides		Free fatty acids Serum
	Heart	Liver	
Control			
1	3.6 ± 1.0 (3)	6.6 ± 1.5 (4)	0.49 ± 0.08 (3)
2	3.9 ± 1.7 (7)	7.3 ± 3.1 (12)	0.61 ± 0.11 (10)
	—	8.5 ± 1.5 (4)	0.63 ± 0.13 (4)
Hypoxic			
50.5 kPa 1	3.3 ± 0.9 (3)	4.5 ± 0.6 (4)	0.61 ± 0.11 (3)
40.8 kPa 2	3.0 ± 0.7 (7)	23.3 ± 14.2^a (12)	0.99 ± 0.22 (10)
Food-restricted controls 1	—	2.3 ± 0.4^b (4)	0.86 ± 0.21 (4)

(Salmon *et al* 1974) Increased liver lactate concentrations occur in hypoxia (Cipriano & Pace 1971) but it does not seem probable that the lactate control of lipogenesis is able to override the lipolytic effects of the sympathoadrenal activity

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Abstract

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An equation of two exponential terms was found to adequately describe the efflux of phenylalanine and tryptophan from cerebral cortex slices of adult and 7-day-old rats. These exponential terms described two forms (components) of efflux taking place at different rates. The fast component of efflux was more prominent in the slices from young rats than in those from adults. In both age groups the contribution of the fast component increased with an increasing amino acid concentration in the superfusate but there were changes in the rate constants. The rate constants of the fast components were equal in all experimental conditions in both age groups. The rate constants for the slow components of both amino acids are low in the slices from adult rats than in those from young rats. The reason for efflux occurring at two different rates is discussed. The fast component probably consisted of the amino acid originating from the extracellular space of the slices and of the intracellular amino acid released by exchange. The slow component consisted of the amino acid released from the cells by other mechanisms, e.g. by diffusion through membranes or by some active efflux processes. The cerebral cortex slices from adult rats have greater ability to concentrate aromatic amino acids than the slices from young rats. This may partly be dependent on the more efficient influx and partly on the slower efflux of amino acids in the slices from adults.

Key words: Cerebral cortex slices, brain development, transport of amino acids, phenylalanine, tryptophan

The properties of amino acid influx into cerebral slices have been much studied (Oja & Vahvelainen 1975). Some of these studies have concerned changes in the influx during ontogenetic development (Vahvelainen and Oja 1972, Vahvelainen and Oja 1975). Little is known about the mechanism of amino acid efflux from the slices and about its possible changes during development.

The efflux of amino acids from adult nervous tissue is assumed to be at least partly an active process. It is saturable in some cases, stereospecific, dependent on temperature and ion concentrations in the medium, there is competition among amino acids transported from the cells, and the efflux rate can be modified by compounds influencing cell metabolism (Levi *et al* 1965, Levi *et al* 1966, Cherayil *et al* 1967, Srinivasan *et al* 1969, Blasberg *et al* 1970, Jones and Banks 1970, Arnfred and Hertz 1971, Cutler *et al* 1971, Hopkin *et al* 1971, Joanny *et al* 1971, Joanny *et al* 1973, Mulder and Snyder

In addition to possible active efflux mechanisms and simple diffusion, a process called exchange may contribute to the efflux of amino acids. Both homoeexchange (transport stimulated by the presence of the same amino acid on both sides of the membrane) and heteroexchange (transport stimulated by the presence of structurally related amino acids on the two sides of the membrane) have been demonstrated in brain slices (Joanny *et al.* 1971; Cutler *et al.* 1971; Battistini *et al.* 1972; Joanny *et al.* 1973).

This paper reports on a study of the efflux of phenylalanine and tryptophan from the cerebral cortex slices of adult and 7-day-old rats and its enhancement by homoeexchange. These amino acids were selected for study for the following reasons. 1) The transport of aromatic amino acids is especially interesting in nervous tissue because of their role as precursors of biogenic amines. 2) Since there are differences in the influx of these amino acids in cerebral cortex slices prepared from adult and young rats (Vahvelainen and Oja 1972), it is possible that also the efflux of these amino acids changes during development. 3) Phenylalanine and tryptophan compete in influx (Vahvelainen and Oja 1975) indicating that they least partly share the same transport mechanism. The efflux mechanisms of these amino acids may therefore also be similar.

Materials and Methods

The radioactive amino acids ^3H -tryptophan- H^3 (7) (sp. act. 1.8 Ci/mmol) and ^3H -phenylalanine-2,3- H^3 (sp. act. 5.0 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Slices of cerebral cortex were prepared from 7-day-old (18–19 g) and adult (180–200 g) Sprague-Dawley rats. The animals were decapitated, their brains excised and briefly rinsed in cold 0.9% saline. The slices were cut at 4°C with a non-wetted tissue slicer of Slade-Ruggs type. The thickness of the slices was 0.5 mm, one side of each slice was the intact brain surface.

Experimental procedure

In each experiment 6 slices weighing in total about 300 mg were preincubated in an Erlenmeyer flask for 10 min under continuous flow of pure oxygen at 37°C in Warburg apparatus and shaken (50 oscillations/min, amplitude 20 mm). The medium was 5 ml Krebs-Ringer phosphate solution (pH 7.4) (Umbreit *et al.* 1964) (final concentrations in mmol/l: NaCl 127, KCl 3.1, CaCl_2 2.7, KH_2PO_4 1.3, MgSO_4 1.3 and Na_2HPO_4 1.3) containing 10 mmol/l glucose, 0.5 mmol/l unlabelled tryptophan or phenylalanine, and μCi of the corresponding tritiated amino acid. The medium was prewarmed and preoxygenated for 15 min before incubating the slices.

The slices were separated from the preincubation medium by filtering and rinsed briefly with 15 ml of cold Krebs-Ringer phosphate solution. Then they were transferred rapidly one at a time into small glass cylinders. The cylinders contained about 2 ml of superfusion medium: Krebs-Ringer phosphate solution with glucose (10 mmol/l) and unlabelled phenylalanine or tryptophan (0.05, 0.5 or 5.0 mmol/l). Two slices were put into each cylinder. These incubation vessels had been previously connected to an electrochemical recording apparatus (Doms, Hendelberg) (Fig. 1). The superfusion medium in the inlet reservoirs, in the silicone tubes and in the incubation cylinders was prewarmed and preoxygenated by bubbling for 30 min. The flow rate of the medium was adjusted to about 1 ml/min. It was measured during 15 min before and after every incubation and varied from 0.9–1.3 ml/min. During the incubation it did not change more than 0.1 ml/min.

The efflux of radioactivity out of the slices was followed for 60 min. The effluents were collected separately from the outlet tubes directly into scintillation counting vials which were changed every 2 min. The volumes of the fractions were calculated from the measured flow rates of the superfusate. The average fraction volume was 2.3 ml (range 2.07–2.60 ml). During incubation the slices were oxygenated, fibroscopically examined in the Warburg apparatus and during preincubation. At the end of incubation the volumes of the effluents in the incubation vessels and in the outlet tubes were measured.

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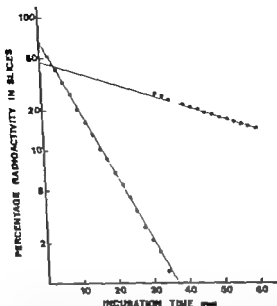


Fig. 2. Method of analysing the efflux curve. The mean values of 6 expts. of [^3H]phenylalanine efflux from cerebral cortex slices prepared from adult rats and superfused with medium containing 5.0 mmol/l unlabelled phenylalanine, presented as an example. (○) Experimental results, per cent of initial radioactivity left in the slices on logarithmic scale plotted against time after beginning efflux. After about 40 min the points can be considered to fall on a straight line. The slow component of the curve is obtained from this line. The fast component (○) is obtained by subtracting the line from the experimentally determined points. The values of the fast component can also be considered to fall on a straight line. The slopes of the two lines represent the rate constants for the two components. The extrapolation of the two lines to time zero gives the percentages of the total initial radioactivity of the components. The final analyses were composed separately for each individual expt. Overall means and standard deviations were also computed. See text for more details.

below f is the radioactivity in the slices (per cent of the total initial radioactivity) at time t (min), F and F_0 are the total initial radioactivities in the two components of efflux (per cent of the total initial radioactivity in the slices), and k and k_0 are the rate constants (min^{-1}) for the amino acid efflux in the two components.

The values recorded during the first 4 min are omitted from the calculations because of the debouring of the released label in the nonradioactive superfusion medium initially present in the outlet tubes (Fig. 3). The total radioactivity in the slices at 6 min was taken as 100 per cent.

The final analysis of the curves were made for each individual expt. in the following way (see Fig. 2). First the slow component was determined by calculating the linear correlation between the incubation time and the logarithmic value of percentage radioactivity left in the slices for the last 5 measurements (at 52, 54, 56, 58 and 60 min). Each measurement was then added one by one to the regression data until the absolute value of the correlation coefficient decreased two times successively. The two poorly fitting values were then discarded from the data and a least-squares line was fitted through the remaining points. The average number of points remaining in the slow component was eight, and the correlation coefficients varied from 0.99999 to 0.9983. The slow component was then subtracted from the experimental values and another least-squares line was fitted through the remaining points to obtain the regression for the fast component. The correlation coefficients for the fast-component lines varied from -0.99915 to -0.96445. The slopes of the lines represent the rate constants for the two components of efflux (k and k_0). The extrapolation of the lines to time zero gives the total initial radioactivities in the two components (F and F_0). The sum of F and F_0 was in all cases little over 100 per cent (about 105 per cent). This was interpreted to be because the calculations were not started leaving some of the fast component in the slow component. Since the errors were small, the corrections were considered unnecessary and the values obtained by

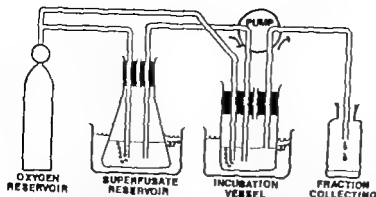


Fig. 1 The experimental setup for the study of amino acid efflux from tissue slices by continuous washout.

After incubation the slices were separated by filter paper, weighed in 1.0 ml of 5% trichloroacetic acid (TCA) (w/v) homogenized and centrifuged for 30 min at 10 000 g . The radioactivity of the TCA-soluble fractions, of the effluent fractions, of the samples from the superfusion medium left in the outlet tubes of the samples from the preincubation medium was measured by liquid scintillation counting (Warriner Nuclear Technology Ltd.) in Aquasol® (New England Nuclear, Dorval, Quebec). The quenching of the samples was determined by the external standard channel ratio method and the efficiency of counting from standard samples.

Chromatographic analyses

In order to check the possible metabolic breakdown of the labelled phenylalanine and tryptophan in the experiments, a number of slices were preincubated as above for 30 min with 10 mCi/l [3 H]tryptophan or [3 H]phenylalanine. Some slices were then superfused for 60 min as above. Each experiment was repeated 4 times. After the incubations the slices were immediately homogenized with 0.5 ml of cold 75% ethanol containing 0.05% edetate and 0.05% ascorbic acid. The homogenates were centrifuged for 30 min at 10 000 g , stored overnight at +4°C and centrifuged again. Samples of 10 μ l from the supernatants were applied on 0.25 mm silica gel thin layer plates (Merck AG) and developed with phenol-water (3:1) and n -butanol-ethanol-25% ammonia (8:1:1 v/v/v). Standard amino acid mixtures were run in parallel and stained with ninhydrin or Ehrlich reagent in order to localize the spots corresponding to phenylalanine and tryptophan in the unstained experimental samples. The portion of the gel containing the chromatographed samples was scratched out in small spots which were suspended in 50% ethanol and stored in Instagel and counted with the liquid scintillation spectrometer. Quenching and efficiency of counting were determined as above.

On the average, in preincubated but not superfused slices 89 per cent of the original radioactivity was recovered as [3 H]tryptophan and 96 per cent as [3 H]phenylalanine representing more than 91 and 96 per cent of the total radioactivity recovered from the thin layer plates, respectively. In preincubated and superfused slices 87 per cent of the original radioactivity was recovered as [3 H]tryptophan and 98 per cent as [3 H]phenylalanine representing more than 96 and 98 per cent of the total radioactivity recovered after chromatography. Standard deviations in these analyses varied from 1 to 4 per cent. Labelled phenylalanine and tryptophan were declared chromatographically 100% pure by the manufacturer; no radioactive impurities were detected by us either. Measurable amounts of radioactive metabolites were not found in the effluents collected from the incubation vessels. These checks do not show any major breakdown of the labelled amino acids during the efflux experiments.

Calculations

The initial radioactivity in the slices was computed as the sum of the radioactivities in the effluent fractions in the medium left in the outlet tubes and in the TCA-sample (the residual tissue radioactivity except the label in the TCA-precipitates). The percentage of the label remaining in the tissue at any time was calculated by subtracting the total radioactivity recovered in the effluent from the initial tissue content.

The concentration of the amino acid in the slices before washout was calculated from the initial radioactivity by assuming that the intracellular amino acid pool were homogeneously labelled during the preincubation and ignoring any metabolic breakdown of the labelled amino acids.

According to preliminary graphic analyses (Fig. 2 is an example) the efflux of the label seemed to be satisfactorily described by the following equation (cf. Bradl's equation (1971):

$$f_t = f_0 e^{-k_1 t} + F e^{-k_2 t}$$

TABLE I Concentrations of labelled phenylalanine and tryptophan in the cerebral cortex slices of rats before washout, and radioactivity left in the slices after washout.

Age of rats	Amino acid and its conc. in superfusion medium (nmol/l)	Conc. of amino acid before wash-out (nmol/kg)	Radioactivity left after wash-out (%)
adult	Phenylalanine 3.0	568 ± 57 (18)	14 ± 3 (6)
	0.5		17 ± 2 (6)
	0.05		23 ± 2 (6)
	Tryptophan 3.0	573 ± 87 (16)	17 ± 4 (6)
	0.5		23 ± 2 (5)
	0.05		31 ± 7 (3)
7-day-old	Phenylalanine 3.0	654 ± 56 (17)	5 ± 2 (6)
	0.5		5 ± 2 (6)
	0.05		10 ± 2 (5)
	Tryptophan 3.0	669 ± 57 (18)	6 ± 1 (6)
	0.5		10 ± 2 (6)
	0.05		16 ± 2 (6)

The slices were preincubated for 30 min at 37°C in oxygen in Krebs-Ringer phosphate medium (pH 7.4) containing glucose (10 mmol/l), [3 H]amino acid (2 mCi/l) and unlabelled amino acid (0.5 mmol/l). The concentrations of the amino acids in the slices with their standard deviations are expressed as nmol/kg of the (wet) weight of tissue. The release of radioactivity into flowing superfusion medium containing varying concentrations of the corresponding unlabelled amino acid was sampled for 60 min. The radioactivity left in the slices after washout was measured, and these means and standard deviations are expressed as percentages of the initial radioactivities. Number of experiments shown in parentheses.

of the components were about equal for both amino acids in the corresponding concentrations. At the concentration of 3.0 nmol/l the slow component contained slightly more than half, at the concentration of 0.05 nmol/l about 80 per cent of the total radioactivity washed out. In the slices from 7-day-old rats the share of the slow component was generally less than in those from adults. When the amino acid concentration in the superfusate was low some difference between the amino acids was found in the slices from young rats: more tryptophan (81 per cent) than phenylalanine (53 per cent) flowed within the slow component (Table II).

TABLE II Percentages of the total initial radioactivities released in the slow (F_s) and fast (F_f) components of amino acid efflux from cerebral cortex slices prepared from adult and 7-day-old rats.

Amino acid and its conc. in superfusion medium (nmol/l)	Adult rats		7-day-old rats	
	P (%)	F (%)	F (%)	F (%)
Phenylalanine 3.0	54 ± 2	46 ± 3 (6)	33 ± 8	67 ± 9 (5)
	0.5	67 ± 5	40 ± 5	60 ± 10 (3)
	0.05	77 ± 3	53 ± 3	47 ± 4 (3)
Tryptophan 3.0	54 ± 3	46 ± 5 (3)	34 ± 6	66 ± 7 (6)
	0.5	66 ± 7	52 ± 8	48 ± 10 (6)
	0.05	93 ± 5	17 ± 4 (4)	81 ± 6 (6)

The slices were preincubated for 30 min with radioactively labelled amino acid (conc. in medium 0.5 nmol/l). Then the release of the radioactivity into flowing superfusion medium containing varying concentrations of the corresponding unlabelled amino acid was measured in two-minute fractions during 60 min. The means percentages and their standard deviations were calculated as described in Materials and Methods. Number of experiments shown in parentheses.

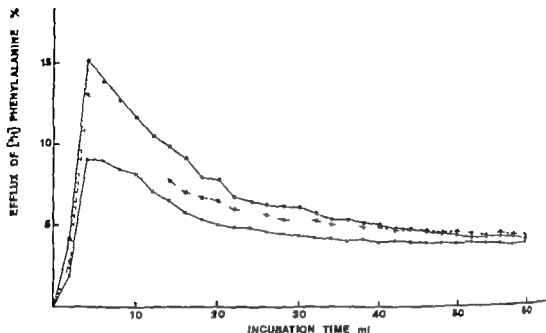


Fig. 3 The efflux of $[^3\text{H}]$ phenylalanine from cerebral cortex slices prepared from adult rats and superfused with media containing 5.0 (●—●), 0.5 (○—○) or 0.05 mmol/l (—×) unlabelled phenylalanine. The slices were preincubated for 30 min at 37°C in oxygen in Krebs-Ringer phosphate solution (pH 7.4) containing glucose (10 mmol/l) $[^3\text{H}]$ phenylalanine (1 mCi/l) and unlabelled phenylalanine (0.5 mmol/l). In order to measure the release of radioactivity the slices were then transferred into the cuvettes in which they were superfused with medium containing varying amounts of unlabelled phenylalanine. Fractions of about 2 ml were collected every 2 min. The results, as means of 6 expts., denotes the percentage of the total amount of label in the slices which is released during 2 min intervals after varying incubation periods. Standard deviations for each point were 5–15% usually not more than 10%.

computation were proportionally adjusted to add up to 100 per cent. The standard deviations for k , $t_{1/2}$ and F were computed for the slopes and intercepts of regression lines (e.g. Snedecor 1959).

The half-time ($t_{1/2}$) of efflux for each component was determined from the equation (e.g. Braden 1971)

$$t_{1/2} = \frac{0.693}{k} \quad (2)$$

where k is the rate constant for the component.

Results

After preincubation the concentrations of phenylalanine and tryptophan were of the same order in the slices from both adult and 7-day-old rats; after washout the radioactivity left in the slices was greater in the adult group (Table I). The efflux of both amino acids into the superfusion media containing varying concentrations of these was rather similar in both age groups: the higher the amino acid concentration in the superfusate the more rapid was the efflux of the label and the less radioactivity remained in the slices after the washout (Table I, Fig. 3).

When the efflux was divided into the two components, it became obvious that the higher the amino acid concentration in the superfusion medium the greater was the contribution of the fast component (Table II), but there were no changes in the rate constants as functions of the amino acid concentration (Table III). In the slices from the adult rats the percentages

used supposes that both the extra- and intracellular compartments are directly connected with the bulk phase. This is an unrealistic assumption (Jones and Banks 1970). However, the idea that the fast component may partly contain the amino acid originating in the extracellular space can be supported by two facts: 1) The share of the fast component was greater in the slices from young rats, and the size of the extracellular space is larger in the cerebral cortex slices from 7-day-old rats than in those from adult rats (Laakso-Oja 1976). 2) The rate constants for all the "fast effluxes" were equal, which can be expected in the case of free diffusion of substances of equal molecular size. Raiteri *et al.* (1971) have analyzed the efflux of glycine and GABA from spinal cord slices and concluded that the fast component of the desaturation curves reflected the washout of the effluent medium and the slow component the loss of isotopes from the tissue as a whole. This interpretation is probably not valid for the present study because of the relatively small contribution of the fast component and because we rinsed the slices after pre-equilibrating them in the radioactive amino acid.

Fertz (1968) studied ion transport in brain slices and inferred that the two components of efflux in his study originated from different intracellular compartments. If both components in this study contain amino acids of intracellular origin, at least two interpretations can be suggested for having two separate components. Firstly, the intracellular amino acids may be located in different compartments, from which the efflux occurs at different rates, and secondly, there may be several processes in the efflux of amino acids at different rates. The validity of the compartment hypothesis is supported also by findings that glutamate is exchanged more rapidly in glial cells than in neurons of cerebral slices from rats (Okamoto and Quastel 1972). If the components of efflux in our experiments originated from different compartments, raising the amino acid concentration in the medium should enlarge the rapidly exchanging compartment. In our view more evidence would be required to support this assumption.

Explaining the components as representing fast and slow efflux processes allows more productive discussion. Because the contribution of the fast process increased with increasing amino acid concentration in the superfusate, it might represent the exchange efflux, and the slow process might consist of other efflux mechanisms. If this interpretation is right, the following conclusions can be drawn: 1) the exchange efflux compared to the other efflux mechanisms may be more prominent in the slices from young rats; 2) the rates of exchange efflux are equal in both age groups, but 3) the other efflux mechanisms are slower in the slices from adults; 4) the rates of efflux of phenylalanine and tryptophan by the slow processes are equal in the slices from young rats, but 5) phenylalanine is released more rapidly than tryptophan in the slices from adult rats.

The slow efflux may comprise passive diffusion of amino acids out of the cells (without exchange) and some active efflux processes. It seems improbable that the constants of the slow processes describe only passive diffusion, because there are differences between the age groups and between the amino acids. The differences between the age groups could also be explained by the more efficient re-uptake of the label in the slices from adult rats. As this seems unlikely, the re-uptake was minimized by using a relatively high flow rate of superfusion (Raiteri *et al.* 1974) and a large medium-volume/tissue-volume ratio (50

TABLE III Rate constants and half times for slow (k_1 , $t_{1/2_1}$) and fast (k_2 , $t_{1/2_2}$) components of amino acid efflux from cerebral cortex slices prepared from adult and 7-day-old rats.

Amino acid and its conc. in superfusion medium (mmol/l)	Adult rats				7-day-old rats			
	$k \times 10^{-3}$ (min ⁻¹)	$t_{1/2}$ (min)	$k_2 \times 10^{-3}$ (min ⁻¹)	$t_{1/2_2}$ (min)	$k \times 10^{-3}$ (min ⁻¹)	$t_{1/2}$ (min)	$k_2 \times 10^{-3}$ (min ⁻¹)	$t_{1/2_2}$ (min)
Phenylalanine								
5.0	21 ± 3	33	134 ± 7	5.2 (6)	76 ± 5	27	150 ± 15	46.0
0.5	22 ± 2	32	137 ± 9	5.1 (6)	31 ± 4	22	141 ± 11	49.0
0.05	19 ± 1	36	146 ± 18	4.7 (6)	28 ± 3	25	130 ± 4	51.0
Tryptophan								
5.0	16 ± 4	4	142 ± 13	4.9 (5)	24 ± 2	29	141 ± 5	49.0
0.5	18 ± 3	39	145 ± 48	4.8 (5)	28 ± 1	25	142 ± 13	49.0
0.05	14 ± 2	49	147 ± 9	4.7 (4)	6 ± 2	27	149 ± 40	47.0

The values of k and k_2 with their standard deviations were calculated for the same expts. as in Table as described in Material and Methods. The values of $t_{1/2_1}$ and $t_{1/2_2}$ were calculated from the rate constant ($t_{1/2} = 0.693/k$). Number of expts. shown in parentheses.

The rate constants of the fast components were of equal magnitude for both amino acids in both age groups, but the rate constants of the slow components were smaller in the slices from adults (Table III) (Student's two-tailed t test gave $p < 0.01$ in all tryptophan expts. and at the concentrations of 0.5 and 0.05 mmol/l in phenylalanine expts.) Between amino acids there were no significant differences in the slices from 7-day-old rats, but in slices from adult rats the rate constants for tryptophan were a little smaller than for phenylalanine ($p < 0.01$ at the concentration of 0.05 mmol/l and $p < 0.05$ at the concentration 0.5 mmol/l). The calculated half-times for the efflux components show a similar picture (Table III). The half times for all the fast components were about 5 min. The half-times for phenylalanine in the slow component were about 34 and 25 min and those for tryptophan about 43 and 27 min in the slices from adult and young rats respectively.

Discussion

The computed efflux curves were practically identical with the experimental curves. The efflux of amino acids from the cerebral cortex slices was thus well described by the equation of two exponential terms. What remains to be clarified is the reason for the two components. Several hypotheses have been previously published mainly suggesting that the components flow from different sites within the tissue or from its surface.

In some discussions mostly concerning the efflux of electrolytes the fast and slow components are considered to be released from extra- and intracellular compartments of the cells respectively (Winograd and Shanes 1962, Zadunavsky and Curran 1963, Ames and Nesher 1966, Borle 1969, Kennedy and Voaden 1974). For the data of the present study this interpretation may not be valid for the following reasons: 1) Increasing the concentration of unlabelled amino acid in the superfusate relatively increased the recovery of the amino acids within the fast component. There is no reason to believe that the extracellular space of the slices enlarged noticeably at higher amino acid concentrations. 2) The mathematical

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Also the fact that the efflux of the label in the slow component did not increase with increasing amino acid concentration in the superfusate, supports the view that the difference in the rate constants were not caused by different rates of re uptake.

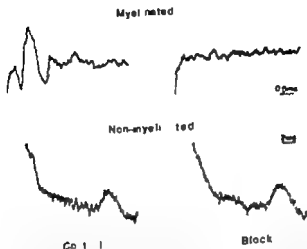
In conclusion, the most probable explanation for the two components of efflux in this study is that the fast component consists of the amino acid released from the extracellular space of the slices and the amino acid released from the cells by exchange, and the slow component involves other efflux processes from within the cells.

The equilibrium concentration of an amino acid in the slices depends both on the influx and the efflux. The cerebral cortex slices from adult rats have been found to concentrate e.g. tyrosine more effectively than the slices from young rats (Vahvelainen and Oja 1968). The maximum velocity of the saturable influx of aromatic amino acids is greater in the slices from adult rats (Vahvelainen and Oja 1972). The present experiments show that the efflux of these amino acids is more rapid from the slices, and probably from within the cells in the slices from young rats. This cannot be proved by the greater share of the fast component in the slices from young rats, because we do not know how much of it originates in the cells. But the smaller rate constants of the slow components in the slices from adults suggest that the slower efflux contributes to the greater ability of the slices from adults to concentrate aromatic amino acids.

The skilful technical assistance of Mrs Pirko Erkkila is gratefully acknowledged. I am also indebted to Mr Esa Korpi, B.M. for his help in chromatographic analyses.

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1 Anodal block of myelinated fibres in the left aortic nerve, as illustrated by its effects on evoked action potentials. Separate oscilloscope sweeps, all with the same vertical sensitivity. Scale characteristics: sweep 0.02 sec, 6 V; baseline 1.5 mV, 6 V. Note that compound A fibres were absent during block, while the compound C wave remained unchanged.

In our first study of the function of baroreceptor C fibres, we examined the contribution of aortic nerve C fibres to pressure-induced bradycardia, by recording changes in heart rate when the aortic nerve was fully intact and when its A fibres had been temporarily blocked by a polarizing current (anodal block). The bradycardia resulting from a moderate rise in pressure was found to be exclusively mediated by baroreceptor A fibres: a rise in aortic pressure of 20–30 mmHg was insufficient to evoke bradycardia via aortic nerve C fibres in the rabbit (Aars and Myrnes 1974). We now report the results of a more comprehensive study of the function of aortic nerve C fibres, in which the reflex responses to a pressure rise were assessed by changes in sympathetic discharges to the kidney. As in the earlier report, fibres in aortic nerve A and C fibres were separated by means of anodal block of the A fibres.

Material and Methods

Rabbits were anaesthetized with 3 ml 1% chloralose and 3 ml 25% urethane per kg: half i.v. half i.p. When necessary, ventilation was supplemented through the right jugular vein. Body temperature was maintained by means of covers and heating lamp. The animals were tracheotomized and respired air. Arterial pressure was measured with Statham P23De transducer connected to catheter in the right common carotid artery. Brief rises of 20–40 mmHg mean pressure in the aortic arch could be produced by inflation of balloon Fogarty Arterial Embolization Cath. 4F) inserted through the left femoral artery and positioned in the ascending thoracic aorta. Sympathetic nerve activity was recorded in the left renal nerve with silver electrodes and differential amplifier (Grass P15 B). The activity was averaged (Copley 9832 A) and recorded on Beckman R 411 Dynograph, together with the blood pressure. Output from the averager was linearly related to the activity found in the renal nerve. The left aortic nerve was dissected in the neck and covered by paraffin oil. Three pairs of silver electrodes (diameter 0.6 mm) were placed on the left aortic nerve for electrical stimulation (Grass SD9) in the caudal part, for anodal block in the middle and cranially for recording of electrical activity in the nerve (Grass P15 B). The anodal block was effected by passing current through the nerve between the electrodes 3–4 mm apart, the current being delivered from specially designed constant current generator. The slope of the rising current was less than 10 μ A/s.

The function of baroreceptor C fibres in the rabbit's aortic nerve

By

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Abstract

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The participation of aortic nerve C fibres in the baroreflex was investigated by recording changes in nerve activity in response to acute increases in arterial pressure, in two experimental situations. In the first, A and C fibres of the left aortic nerve intact, and with the A fibres temporarily blocked by a hyperpolarized current (anodal block). Pressure was increased by manual inflation of an intra-aortic balloon, and interference from other baroreceptor areas was avoided by carotid occlusion and sectioning of the right aortic nerve. Rises in mean arterial pressure exceeding 20 mmHg, at levels above 110 mmHg, were needed to trigger sympathetic inhibition via C fibres. A rise of 45 mmHg caused 90% reduction in sympathetic activity equal to that obtained by stimulation of aortic nerve C fibres at about 3 Hz. In contrast, a rise of 20-30 mmHg evoked more than 60% reduction in sympathetic activity when the A fibres were operative. Judged by these studies of peak reflex responses to brief pressure rises, baroreceptors with C fibres in the aortic nerve have a much higher threshold to pressure than their myelinated counterparts: the C fibres contribute to the baroreflex inhibition of sympathetic discharge only when pressure is increased well above normal resting levels.

Despite the existence of a high proportion of non-myelinated baroreceptor afferents (C fibres) in the aortic and carotid sinus nerves, the knowledge of baroreceptor function is almost completely restricted to receptors with myelinated (A) fibres (Heymans and Ne 1958, Kirchheim 1976). All that is known of the C fibres, is that when electrically evoked activity in these fibres will induce severe reflex bradycardia, sympathetic inhibition and a fall in blood pressure (Douglas and Ritchie 1956, Douglas, Ritchie and Schaumann 1956, Douglas and Schaumann 1956, Kendrick and Matson 1971, Ninomiya, Nishimaru and Iwasawa 1971, Koizumi and Sato 1972, Kardon, Peterson and Bishop 1973, 1975). Response characteristics of the C fibre receptors, and hence their contribution to the baroreflex, remain virtually unknown.

Preliminary presentations of this paper were given in June 1976 at the 7th European Congress of Cardiology, Amsterdam, The Netherlands, and at the 8th Nordic Hypertension Meeting, Marstrand (Aars, Myhre and Haswell 1976).

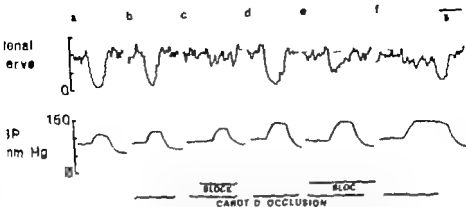


Fig. 3. Reflex responses of average sympathetic activity in ventral nerve to balloons-occluded aorta in arterial anaesthesia. Ischaemia from right aortic nerve and right carotid sinus occluded. With left aortic and vagus nerves intact. *b-f* Afferent baroreceptor pathways restricted to the left aortic nerve by transitory occlusion of left carotid artery. Responses to moderate (*b-c*) and larger (*d-e*) rises of pressure 1 s periods (anodal block (*f*)). Aortic baroreceptors were functionally connected to the brain through aortic C fibres only: baroreceptors in records *b-d* of both A and C fibres could transmit action potentials. The figure shows the threshold for excitation of receptors. With aortic C fibres was exceeded in records *d* but not readily reached by the pressure rises as in *b*. Record *f* shows the obligatory test ending all experiments: the recording of sympathetic effects of pressure rise after the left aortic nerve had been cut. A slight response persisted, which was subsequently found to result from excitation of vagal afferents. The sharp decrease caused by activation of carotid sinus receptors (release of carotid occlusion) served as a final check on the integrity of central pathways of the reflex. Same animal as in Fig. 2.

as mediated by pressure-induced activation of aortic nerve C fibres—and that the C fibres had probably contributed to the reflex response shown in Fig. 3 *d*, with the aortic nerve intact.

The higher threshold for activation of receptors with C than A fibres was confirmed in all rabbits (Fig. 4). A 20–30 mmHg rise in pressure, corresponding to the threshold of C fibre reflexes, caused a 60% decrease in sympathetic discharge when receptors with A fibres were allowed to participate. Related to maximum mean pressure (Fig. 4 right), aortic nerve C fibres contributed to the sympathetic inhibition only at pressures exceeding 110 mmHg, whereas with A fibres operative, the inhibition was almost complete from 100 mmHg upwards.

In an attempt to assess the firing frequency of aortic C fibres during the reflex events depicted in Fig. 4, we recorded the sympathetic responses to electrical, combined stimulation of A and C fibres in the left aortic nerve (Fig. 5). The stimulation was usually performed about simultaneous occlusion of the left carotid artery and in a frequency range where selective activation of A fibres is known to be devoid of reflex effects (Douglas *et al.* 1956). Thus as tested and confirmed in present rabbits. Accordingly although the results plotted in Fig. 5 were obtained by combined A and C fibre stimulation, they represent reflex effects of activity in C fibres alone. Pulses of 4 Hz were found to produce a 65% reduction in sympathetic discharge, while maximum effects were obtained by about 10 Hz. These responses were not altered by concomitant carotid occlusion or anodal block of the A fibres. Electrical stimulation or pressure increases produced only negligible changes in heart rate. Injection of noradrenaline (5 μ g/kg min) initially led to a rise in pressure and inhibition of

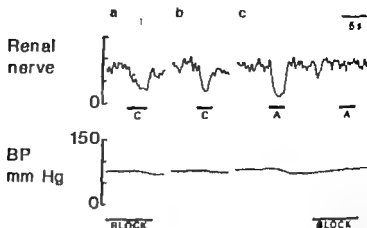


Fig. 4. Specificity of the anodal block of A fibres in left aortic nerve demonstrated by recordings of reflex changes in average renal nerve activity. Stimulation of aortic nerve distal to blocking site, with pulses 1.5 ms, 6 V 20 Hz (marked C) and 0.02 ms, 6 V 100 Hz (marked A). C fibre stimulation depressed renal nerve activity to the same level with as without anodal block, while the effect of A fibre stimulation is abolished by the block.

An ideal block usually obtained with 5–15 μ A, was characterized by complete and immediate block of conduction in A fibres and no inhibitory or excitatory effects on the C fibres. Higher currents blocked both types of fibres. The block was frequently controlled, and when necessary adjusted, by studying the multi-compound potentials in the aortic nerve (Fig. 1) and the reflex changes in the renal nerve activity (Fig. 2) resulting from stimulation of the aortic nerve. The nerve was stimulated with pulses of 0.02 ms, 6 V at 100 Hz, or 1.5 ms, 6 V 0.5–20 Hz, for excitation of A fibres or A and C fibres combined, respectively. Recorded aortic nerve activity was displayed on an oscilloscope and monitored with a loudspeaker.

The actual experiment consisted of recording the peak sympathetic responses to a 5–10 s rise in mean pressure in two different situations: with the left aortic nerve intact, and with conduction through the A fibres blocked. Reflex effects via baroreceptors of the left carotid sinus were avoided by transitory occlusion of the left common carotid artery during the pressure rise. The right carotid artery was permanently occluded by the arterial catheter, and the right aortic nerve was cut. Duration of the blocking periods never exceeded 20 s. As conduction in the A fibres recovered immediately or within a few seconds after cessation of the block, several series of these experiments, with different increases in pressure, could be performed in each animal. In some rabbits, attempts were made to repeat the procedures during i.v. infusion of an adrenaline. Blood pressure was then maintained close to control levels by bleeding the animals. At the end of all experiments, we recorded the sympathetic response, if any, to a pressure rise during carotid occlusion after the left aortic nerve had been cut.

These studies were made in a considerable number of rabbits. Results presented in this paper are from 12 rabbits fulfilling the following criteria: resting mean arterial pressure between 70 and 109 mmHg, an anodal block frequently controlled, and no alterations throughout the experiment in sympathetic response to aortic nerve stimulation or a rise in pressure.

Results

Typically a 30–35 mmHg rise in mean arterial pressure caused severe inhibition of sympathetic discharge when aortic A and C fibres were intact, but a barely noticeable drop when the A fibres were blocked (Fig. 3 b, c). Sympathetic inhibition occurred however when during anodal block of A fibres the pressure was increased by about 50 mmHg (Fig. 3). The immediately preceding demonstration of selective block of the A fibres (Fig. 2a) and the subsequent diminutive response to the same pressure rise after the left aortic nerve had been cut (Fig. 3 d), indicated that the reflex sympathetic discharge shows

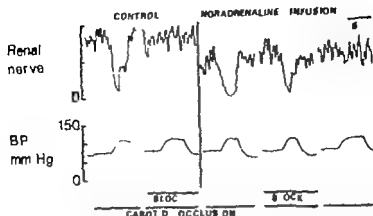


Fig. 6. Effect of noradrenaline ($5 \mu\text{g/kg min}$) on threshold for excitation of baroreflex via aortic nerve C fibres. Procedures as in Fig. 3. Note reduced sympathetic activity despite equal pressure (obtained by occluding) during infusion of noradrenaline, and reflex sympathetic inhibition by a pressure rise which prior to the infusion had no effect on C fibres. The last recording shows the lack of effect after the left aortic nerve had been cut.

Discussion

Our results demonstrated that a larger rise of pressure was needed to elicit reflex sympathetic inhibition via aortic nerve C than A fibres. Most likely this means that receptors with C fibres have higher thresholds than receptors with A fibres. At least in theory, however, receptor threshold and central nervous responses to C fibre activity might have been modified by the block of efferent or afferent traffic in the A fibres, by carotid occlusion, or by the anaesthesia. Efferent stimulation of the aortic or sinus nerves has been shown not to affect baroreceptor activity in these nerves, but the investigation was probably limited to receptors with A fibres (Neal and O'Reagan 1971). As for influences on central pathways of the C fibres by cessation of afferent activity in baroreceptor A fibres, our findings of equal responses to stimulation of C fibres before, as during carotid occlusion or anodal block suggest that such interactions were not of importance. The carotid occlusion might have activated carotid chemoreceptors, thereby interfering with the baroreflex (Körner *et al.* 1973, Ott and Shepherd 1973, Poffeier and Shepherd 1975). However the occlusion was probably too brief for this to happen (Poffeier and Shepherd 1975), since neither sympathetic activity nor blood pressure showed signs of secondary increases during the occlusion, and—as just mentioned—responses to stimulation of the aortic nerve remained unaffected. A number of anesthetic agents are known to increase baroreceptor sensitivity but the studies have all been limited to A fibres (Price and Widdicombe 1962, Blacoe and Millar 1964, Hagenaue, Petach and Arndt 1976). Different effects on A and C fibre receptors, like that described for pulmonary high- and low-threshold stretch receptors (Coleridge *et al.* 1968), cannot be totally excluded, the same applies to the central pathways of the two types of fibres (Blacoe and Millar 1966). On the whole, however we believe that the experimental anaesthesia did not modify influence our results.

vascular resistance in cats is known to change in parallel with systemic arterial

Sympathetic discharge

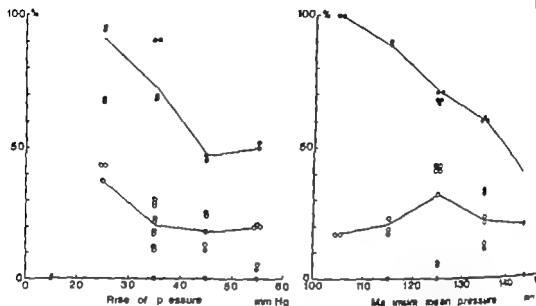


Fig. 4 Reflex reductions in renal sympathetic discharge mediated by aortic nerve C f fibres (filled symbols) or A and C f fibres (open symbols) in response to a 5–10 s rise in mean arterial pressure. Left: Related to magnitude of pressure rise. Right: Related to maximum pressure reached by the rise. Resting pressure 70–109 mmHg. All observations from 10 rabbits each animal represented by its mean value of all observations for each 10 mmHg step. Lines are medians.

sympathetic activity. The nerve activity remained lower than normal when, after a few minutes, blood pressure had been adjusted to pre-infusion levels. Rises of pressure too small to inhibit sympathetic discharge through activation of C fibres before the infusion caused a marked sympathetic depression (Fig. 6).

Sectioning of the left aortic nerve usually abolished the sympathetic response to a rise in pressure during carotid occlusion (Fig. 6). A small sympathetic reduction was occasionally seen (Fig. 3 f), but this developed more slowly than before, and disappeared after the vagus nerves had been cut.

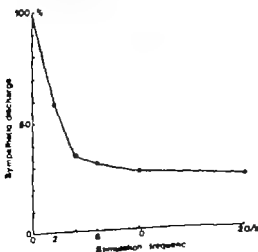


Fig. 5 Renal nerve responses to electrical stimulation of A and C f fibres in the whole left aortic nerve (stimulus, 6 V in periods of 5 s). Control mean pressures 70–109 mmHg. Left carotid sinus occluded. Fluorescence from right aortic nodal sinus period included. The media of observations from 10 animals with 95% confidence intervals shown by shaded area.

with Nembutal, a 20-30 mmHg increase in systolic pressure was found to be insufficient for elicitation of reflex bradycardia via aortic nerve C fibres (Aars and Myhre 1974). Judged by the present results, this was to be expected, since the pressure stimulus must have been too low to trigger activity in the C fibre receptors.

The extreme sensitivity of sympathetic neurons to electrical stimulation of aortic nerve fibres (Fig. 5) agrees well with observations by other authors. Thus, Niimiya *et al* (1971) found that bilateral stimulation of aortic nerve C fibres in cats at 2 impulses/heart beat (equal to about 4 Hz) caused a 50% reduction in average renal nerve activity 8-16 s after start of stimulation. A similar effect was observed in sympathetic fibres to skeletal muscle upon stimulation of the vagodepressor nerve, also in cats (Kotzumi and Sato 1972). Blood pressure responses to stimulation of C fibres in the rabbit's aortic nerve reached 60% of maximum at 4 Hz (Douglas *et al* 1956). That is almost identical to the effect of the same stimulation on activity in the renal nerve (Fig. 5).

A strict comparison between sympathetic responses to pressure-induced and electrically evoked activity in aortic nerve C fibres is probably not warranted, since the aortic nerve may contain fibres from receptors outside the aortic arch, e.g. from the heart (Sarkar 1922, Douglas and Ritchie 1956). Also the pattern and central impact of individual discharge evoked by the pressure rise most likely differed from that evoked by electrical stimulation of all C fibres in the nerve. With due respect to these reservations, however, our results could seem to suggest that, on the whole, i.e. C fibres of the rabbit's aortic nerve have no or negligible spontaneous activity at normal arterial pressures, ii, their receptors have thresholds at about 30 mmHg for abrupt rises in mean pressure and above 110 mmHg for steady mean pressure, and iii, their average discharge rate is low, probably less than 5 impulses per mean pressure up to 150 mmHg. Lundgren (1952), who was the first to study in detail the characteristics of thin and thick baroreceptor fibres, found that the "small spike fibres"

the cat sinus nerve had thresholds for steady pressures at 120-150 mmHg, as compared to 80-120 mmHg for the "large spike fibres". Very likely these were non-myelinated and myelinated fibres, respectively. The difference in threshold between the two has later been suggested or confirmed by others (Fidone and Sato 1969, Coleridge *et al* 1973 a). Thorén, Sævi and Brown (1977 a) presented the first study of individual baroreceptor C fibres, in which the fibres were grouped according to conduction velocities and type of firing. Utilizing controlled perfusion of the aortic arch, they found that, when tested by a pressure ramp, the threshold for the most commonly occurring type of C fibres in the rat's aortic nerve was 152 mmHg (range 85-260). From our deductions, we would expect about the same to be found in the rabbit.

In the one rabbit successfully studied, noradrenaline induced a pressure-independent depression of sympathetic activity and a reduction in pressure threshold for receptors with aortic nerve C fibres. These findings support the conclusions of previous studies in this laboratory (Åkre and Aars 1977), in suggesting that the C fibre receptors constitute the target and the means of efferent sympathetic control of the baroreflex. Similarly Thorén *et al* (1977) observed that addition of noradrenaline to the aortic arch perfusate led to lowering of the threshold of individual aortic nerve C fibres. Unfortunately most of our attempts at studying the effect of noradrenaline on the reflex failed, due to deterioration of

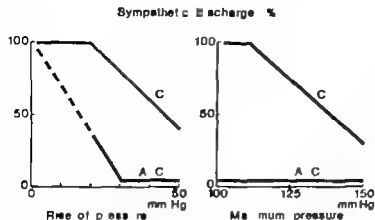


Fig. 7 Participation of aortic nerve A and C fibres in the baroreflex control of renal nerve activity, as elicited by a 5–10 s abrupt rise in mean aortic pressure. Schematic presentation based on Fig. 4 after subtraction of pressure-independent activity

pressure and vascular resistance in skeletal muscle during elevation of carotid sinus pressure up to 200 mmHg (Kendrick, Öberg and Wennergren 1972, Little, Wennergren and Öberg 1975). Whether this balance is limited to the activation of A fibres with different receptor thresholds, or if it holds for baroreceptor C fibres as well is presently largely unknown. Ninomiya *et al* (1971) found that the stimulation of aortic nerve C fibres in cats reduced sympathetic activity to the spleen and the kidney to about the same degree. In rabbits, we have often observed a closer correlation between the reductions in renal nerve activity and blood pressure during stimulation of aortic nerve C than A fibres. We certainly have never seen indications of an arrangement of the sympathetic supply to the kidney similar to that of the cardiac sympathetic neurons, which have been found to be without projections from baroreceptor C fibres (Kardon *et al* 1975).

In order to arrive at a simpler presentation of the results plotted in Fig. 4 we have in Fig. 5 subtracted the baroreceptor independent activity which remained during maximum responses to A and C fibre activity (Fig. 4 and 5) and straightened the lines slightly. Fig. 5 then presents, in a schematic way, the contribution of aortic nerve A and C fibres to the baroreflex control of sympathetic vasomotor neurons projecting to the kidney. Assuming—as just discussed—that changes in renal sympathetic activity are fairly typical of the baroreflex, Fig. 7 would apply to the control of vasomotor activity also to other vascular beds engaged in the baroreflex. It is important to realize that this illustration of the reflex is valid only for peak sympathetic responses to a pressure step. The reflex effects undoubtedly depend upon the rate of rise of pressure but this unfortunately could not be systematically studied with the present technique. Instead we tried to keep the rate of rise constant. This often proved impossible during infusion of noradrenaline, when, due to volume depletion and reduced cardiac output, the rate of pressure rise became very low. High steady pressures, which would have been better suited for determination of thresholds, were difficult to obtain, especially in combination with the desired steep rising phase. Also occlusion of the descending thoracic aorta in rabbits after about 30 s is apt to induce a secondary marked increase in efferent renal nerve activity (Aars and Akre, unpublished results).

Influence on the heart rate of pressure-induced activity in A and C fibres, respectively, could not be determined in the present experiments, due to the vagolytic effect of chloral urethane (Körner, Uther and White 1968). However, in our earlier studies in rabbits

and with Nembutal, a 20–30 mmHg increase in systolic pressure was found to be insufficient for elicitation of reflex bradycardia via aortic nerve C fibres (Aars and Myhre 1974). Judged by the present results, this was to be expected, since the pressure stimulus must have been too low to trigger activity in the C fibre receptors.

The extreme sensitivity of sympathetic neurons to electrical stimulation of aortic nerve fibres (Fig. 5) agrees well with observations by other authors. Thus, Nishimiya *et al.* (1971) found that bilateral stimulation of aortic nerve C fibres in cats at 2 impulses/heart beat equal to about 4 Hz caused a 50% reduction in average renal nerve activity 8–16 s after start of stimulation. A similar effect was observed in sympathetic fibres to skeletal muscle upon stimulation of the vagodepressor nerve, also in cats (Kozumi and Sato 1972). Blood pressure responses to stimulation of C fibres in the rabbit's aortic nerve reached 60% of maximum at 4 Hz (Douglas *et al.* 1956). That is almost identical to the effect of the same stimulation on activity in the renal nerve (Fig. 5).

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the preparations late in the day and difficulties in producing pressure rises similar to those obtained prior to infusion of noradrenaline.

Complete functional separation of A and C fibres in the aortic nerve was crucial to the present experiments. Judged by the effects of the block on evoked action potentials (Fig. 1) and on sympathetic responses to aortic nerve stimulation (Fig. 2), such a separation was reached in all experiments presented in this paper. Our main difficulties with the block were electrical damage to the nerve and as reported by others (Coleridge *et al.* 1973 b, Sant Ambrogio and Zimmermann 1973, Fukushima, Yahara and Kato 1975, Thorén, Shepherd and Douglas 1977) excitation of C fibres. These complications were virtually abolished by improvement of electronic equipment in particular by avoidance of electrical noise in the blocking circuit. The slow rise of the current as well as the much lower currents required for anodal block of A fibres in the aorta than e.g. the vagal nerves (Trenchard 1970, Sant Ambrogio *et al.* 1972, Coleridge *et al.* 1973 b, Thorén *et al.* 1977 b, Kardon *et al.* 1975) were probably of importance. The occurrence of a frequency-dependent block of C fibres, shown to occur during anodal block of vagal A fibres in cats (Thorén *et al.* 1977 b) was assured against accepting only anodal blocks which did not affect the sympathetic response to C fibre stimulation at 10–20 Hz frequencies well above the expected spontaneous discharge rate in the C fibres. Failure to meet this requirement led to rejection of many experiments. However, with experience, the anodal block in our hands proved a useful and safe, although laborious and consuming tool which enabled us to separate, for the first time, the contribution of aortic nerve A and C fibres to the pressure induced baroreflex inhibition of sympathetic activity.

This work was supported in part by Einar Langfeldt og hustru's Fond.

Not added in proof: A study of the characteristics of aortic baroreceptor C fibres in the rabbit, based on single fibre recordings, was recently published by Thorén and Jones (*Acta physiol. scand.* 1977 99 443–4).

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Tissue osmolality in intestinal villi during luminal perfusion with isotonic electrolyte solutions

By

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Abstract

JODAL, M., D. A. HALLBÄCK and O. LUNDGREN *Tissue osmolality in intestinal villi during luminal perfusion with isotonic electrolyte solutions* Acta physiol. scand. 1978. 102. 94-107

A cryoscopic technique has been developed that makes it possible to determine tissue osmolality in the core of the intestinal villi. During absorption from an isotonic electrolyte solution containing glucose, an osmolality gradient was demonstrated from tip to base of the villi in both the jejunum and the ileum. The tissue osmolality at the villous tips was measured at 1 000-1 200 mOsm/kg H₂O while the osmolality at the villous bases was approximately isotonic with plasma. Increasing intestinal blood flow by administration of a vasodilator drug, or making the intestine ischemic by clamping the intestinal vessel supply while supplying the mucosa with oxygen, markedly decreased tissue osmolality. Substituting sodium ions with choline in the luminal perfusate abolished almost completely the tissue hyperosmolality and the intestine became a secretory organ. These observations are consistent with the view that the observed villous tissue hyperosmolality was created by a countercurrent multiplication of sodium chloride. The physiological implications of this mechanism are discussed and it is, among other things, proposed that the hyperosmolar region represents the hyperosmotic compartment necessary for explaining water and electrolyte absorption.

The vascular arrangement of intestinal villi is consistent with the existence of a countercurrent exchanger (for reviews, see Lundgren 1967, 1974). In the cat the exchanger is formed by an arterial vessel running in the central part of the villous core and by an extensive network of subepithelial capillary vessels surrounding the artery. It was proposed by Jodal and coworkers (Haljamäe *et al.* 1973, Jodal 1973) that the most important physiological implication of the exchanger was its significance for water and sodium absorption by creating the hyperosmolar compartment necessary to explain water absorption in the absence of, or against, a lumen to plasma osmolality difference (see Curran and McIntosh 1962, Schultz and Curran 1968). This is accomplished if the exchanger acts as a countercurrent multiplier in a similar way as in the kidney papillae.

In the cited study by Haljamäe *et al.* (1973) evidence for the countercurrent hypothesis was obtained by determining the sodium concentration in the villous core and in the lumen. The data showed that the sodium concentration in the villous core was higher than in the lumen, and that the difference was proportional to the osmolality difference. This is consistent with the hypothesis that the villous core represents a hyperosmotic compartment necessary for explaining water and electrolyte absorption.

tissue sodium per unit weight of tissue protein, along the length of the villus. These data revealed that the villous tip contained 3-4 times as much sodium per unit weight of protein as the villous base implying that a considerable hyperosmolality may exist at the tip portion of the villus.

The present study was carried out to obtain more direct evidence for the hypothesis proposed by Jodal *et al*. It describes a cryoscopic technique that makes it possible to determine tissue osmolality along the intestinal villi of the feline gut (length 0.7-1 mm). The results obtained with this technique, described below provide strong support for the unidirectional multiplication hypothesis.

Methods

Operative procedures

All experiments were performed on cats anesthetized with chloralose (30 mg/kg *ib*) after a light ether preanesthesia. The animals had been deprived of food for 24 h and had no obvious signs of intestinal activity. The operative procedures as well as the recordings of blood flow and of net water absorption are similar to those described by Jodal *et al.* (1975). Briefly, total viscous outflow from one or two mechanically cannulated intestinal segments, either from mid-jejunum and/or from ileum, as recorded on a drop recorder used operating an occluder near Meckel's arterial blood pressure was measured from the left femoral artery by a pressure transducer (Statham P23AC). Cholinergic influences were eliminated by atropine administration, 1 mg/kg *ib*. In some experiments intestinal vasodilatation was achieved by close infusion of isopropyl noradrenaline (20 μ g/ml) at a rate of 5-10 μ g/min via cannula in the wall branch of the superior mesenteric artery. In one series of experiments, where net water absorption was not monitored (see below), small jejunal incisions were opened along the mucosum border by thermocautery and mounted on metal frames as described by Halperin *et al.* (1973). By means of plastic frames, small baths were then created over the exposed mucosa. The bath was flushed at a constant rate of about 1 ml/min. The temperature of the bath was controlled by thermocouple thermometer (Electrolab, Copenhagen) and kept at 38°C by a heating pad. Initially the mucosa was flushed for 30 min with choline-saline solution (see below). The intestinal segment was then made ischemic by clamping its vascular supply and the solution flushing the mucosa was simultaneously changed to Krebs-glucose solution (see below). In this situation oxygen was allowed to diffuse into the tissue via the mucosal solution by bubbling the solution with oxygen.

Recording of intestinal net water transport

Intestinal net water transport was continuously measured with a recirculating system that was coupled to the lumen of the intestinal segments and contained a large reservoir (volume 1000 ml) to minimize fluctuations. The change of volume of this system was recorded with a volume transducer connected via a tube to the perfusion system. Provided no mobility occurred the recorded change of volume reflected net water absorption or secretion (see Jodal *et al.* 1975). The intraluminal pressure of the outflow end of the segments was kept at 1 cm H₂O and the intestinal segment was perfused at a constant rate of about 1 ml/min by means of a roller pump (Model 800-4, Iwatec Co, Zurich, Switzerland). The temperature of the perfusate entering the segment was continuously monitored with a thermocouple thermometer (Electrolab, Copenhagen) and kept at 38°C by a heating pad.

Cryoscopic technique

Net cryoscopy has earlier been used particularly in the kidney. The reports by Wirtz *et al.* (1951) and many *et al.* (1971) have been of great help in developing the present method for the gut.

General procedures. After at least 20-25 min of constant net water transport or after 45 min incubation in the opened solutions, the segments were rapidly excised and immediately frozen in isopentane, precooled to -160°C in liquid nitrogen. The intestinal wall was then cut obliquely in cryostat kept at -20 to -25°C so as to give cross-sections at different levels of the villi. The tissue sections (10-15 μ m) were embedded in Kerosene between glasses (20-30 \times 0.8 mm) and transferred to a temperature-controlled room kept at -10°C. This unit consisted of an aluminum block (100 \times 30

TISSUE OSMOLALITY

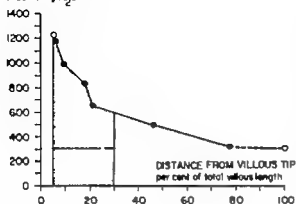
mOsm/kg H₂O

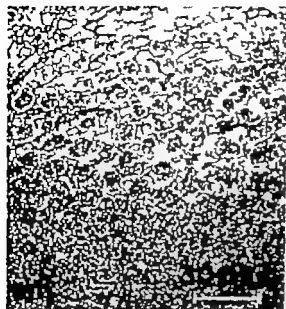
Fig. 1 The computer treatment of the osmolality measurements made on an intestinal section. The actual observations are indicated by filled circles. The line connecting the two points indicates the tissue osmolality as extrapolated to the 5% level of villous length (unfilled circle). Furthermore the osmolality at the base (unfilled circle) was assumed to be that of plasma (or 312 mOsm/kg H₂O also indicated by the horizontal, dotted line). Shaded part of the figure denotes the area under the curve from 5 to 30% of total villous length, which as well estimate mean osmolality in this part of the villus (see Table 1).

15 mm) furnished with a hole (diameter 8 mm) into which a plastic cylinder was tightly fitted with grease. The aluminium block was insulated with Frigolite® 3 mm thick except for a rectangular area over the hole into which the slides fitted. The temperature of the aluminium block was controlled by a sensor system and could be set at any temperature between -4.5 and $+0.5^{\circ}\text{C}$. The whole system was placed on the objective table of a Zeiss standard microscope so that a part of the transilluminated tissue section could be focused and photographed in the microscope. A thin layer of silicone-oil was placed between the aluminium block and the slides to assure good contact and thus a homogeneous and constant temperature of the tissue section. Thawing of the intestinal tissue was achieved by increasing stepwise the temperature, usually in 3–6 steps with at least a 10 min equilibration period between each step. The error of thawing at each temperature was then determined from photographs taken at the end of the equilibration period as will be described below.

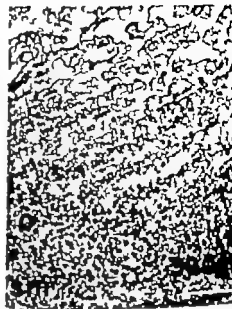
Calibration. The temperature of the aluminium block could be adjusted between $+0.5$ to -4.5°C with a potentiometer and the readings of this was linearly related to the temperature of the aluminium block. Since tissue temperature could not be assumed to be identical to that of the metal blocks, the microscopic system was calibrated. This was done in two principally different ways. First, the melting points of solutions of different osmolalities were determined when the solutions were frozen either in 10 layers between cover glasses at -10°C or as cylindrical blocks (1 mm diameter) in isopentane precooled with liquid nitrogen. In the latter case albumin was added to the solution to facilitate the cutting of the 15 μm thick sections. Second, small everted intestinal rings from rats were incubated for 60 min in Krebs solution in which the osmolalities were varied between 300 and 1000 mOsm/kg H₂O by adding appropriate amounts of mannitol. To avoid changes in osmolality of the incubation medium, large volumes (around 700 ml) were used in each bath. The baths were bubbled with gas containing 5% CO₂ in O₂ and the temperature was kept at $21-23^{\circ}\text{C}$. After the incubation period the intestinal segments were quickly frozen in liquid nitrogen and cut at -20 to -23°C in 10 μm thick sections. The thickness of the sections was then treated exactly as the intestinal segments (see below).

Determination of tissue osmolality. The temperature at which the last ice crystal thawed in a villous cross-section was taken as the tissue melting point. The microscopic magnifications used (17 or 75) were too low to allow any observation of intracellular ice crystals in the villous tissue. Thus, the melting point determined in this study is considered to be a measure of the extracellular osmolality.

The thawing of the intestines, obtained during *in vivo* conditions, always started at the villous tip and gradually spread downwards until, finally the crypt region, the submucosa and the muscularis reached. The percentage of villous tissue melted at each temperature was determined from the photographs and plotted, illustrated in Fig. 1. Usually 3–6 readings were obtained on each section, the tip always normally falling within the tip 5–15 per cent of the villous length. The data were treated in a computer in the way indicated in Fig. 1. The line connecting the two values was extrapolated to the 5 per cent level. Furthermore the base osmolality was assumed to be equal to that of plasma (around 312 mOsm/kg H₂O). From this curve the computer calculated the mean osmolality at 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent of villous length as the mean.



a



b

Fig. 3 *Left panel* Tissue section from a jejunal segment exposed to an isotonic Krebs-mannitol solution, photographed at a temperature corresponding to a freezing point depression caused by solution with an osmolality of 1 160 mOsm/kg H_2O . The crypt region (c) and the submucosa (sm) are frozen. The villous cross-sections close to the intestinal lumen (upper part of the Fig.) are completely thawed. *Right panel* The same tissue section photographed at a temperature corresponding to a freezing point depression caused by a solution of 660 mOsm/kg H_2O .

The thawing of the intestinal wall always started at the villous tip and proceeded toward the base as the tissue temperature was stepwise increased. This is illustrated in Fig. 3 which is taken from an experiment where the lumen was perfused with a Krebs-mannitol solution. In the left panel of Fig. 3 the temperature of the tissue corresponds to a depression of the freezing point that is caused by a solution with an osmolality of 1 160 mOsm/kg H_2O . It is clearly seen that only cross-sections at the very tips of the villi are thawed, while the submucosa, the crypt region and the basal half of the villi are still frozen. Ice crystals are also observed in the intestinal lumen surrounding the thawed cross sections. In the border line zone between melted and completely frozen tissue the ice crystals in the cross-sections are enlarged and reduced in number (Fig. 3).

A change of tissue temperature to a value equivalent to an osmolality of 660 mOsm/kg H_2O resulted in a further thawing of the villi as showed in the right panel of Fig. 3. Here ice crystals are observed only in the basal parts of the villus, in the crypt region and in the mucosa-muscularis. The ice crystals in the intestinal lumen adjacent to the villous cross-sections appear to melt at a somewhat higher temperature than the corresponding cross-section. When tissue temperature was increased to or above that corresponding to a freezing point depression caused by 310 mOsm/kg H_2O the whole tissue section including the submucosa and the muscularis, melted.

Thus, the results of Fig. 3 strongly suggest that there exists a tissue region at the villous tip which has a considerably higher osmolality than that of the fluid perfusing the

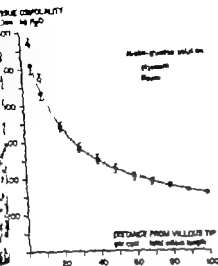


Fig. 4

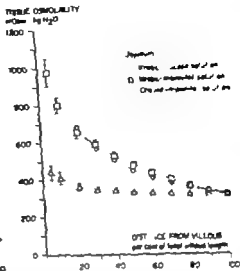


Fig. 5

Fig. 4 Tissue osmolality in villi from jejunum (n = 14) and ileum (n = 14) segments during absorption from isotonic Krebs-glucose solution during resting blood flow. Bars denote S.E.

Fig. 5 Tissue osmolality in villi of the jejunum and ileum perfusing the gut lumen with three different isotonic isotonic intestinal blood flow was taken the resting state. When exposed to the choline-mannitol solution the jejunal segment secreted fluid into the lumen. Bars denote S.E. Number of observations, see table 1

men or that of plasma. Moreover there exists an osmolality gradient within the villus from tip to its base.

Fig. 4 illustrates the computed data for the osmotic gradient along the villus in both jejunum and ileum when the lumen was perfused by an isotonic Krebs-glucose solution. The tip osmolality was estimated to 1139 ± 31 mOsm/kg H₂O ($x \pm S.E.$, n = 14) in the jejunum and to 1026 ± 47 mOsm/kg H₂O (n = 14) in the ileum. These values differ significantly from each other while no significant difference could be demonstrated for the rest of the values illustrated in Fig. 4. Mean osmolality values for different villous sections in the jejunum and the ileum (see Methods) are given in Table 1.

The efficiency of a sodium countercurrent multiplier depends, among other things, on the rate of active sodium transport and the magnitude of certain circulatory parameters in the villi. Fig. 5 and 6 illustrate the effects of reducing the rate of sodium absorption on the villous osmolality gradient in jejunum and ileum. The decrease in sodium absorption was accomplished in two ways, i.e. either by exchanging glucose with mannitol in the luminal perfusate or by replacing all the sodium in the perfusate with choline. The elimination of glucose in the perfusate reduced the villous tip osmolality to 975 ± 72 mOsm/kg H₂O (n = 10) in the jejunum and to 979 ± 70 mOsm/kg H₂O (n = 9) in ileum, the reduction being statistically significant in the jejunum but not in the ileum. Exposing the intestinal mucosa to an isotonic solution containing no sodium ions (choline-mannitol) reduced the villous tip osmolality drastically to 442 ± 39 and 391 ± 24 mOsm/kg H₂O in the jejunum

TABLE I Mean tissue osmolality in mOsm/kg H₂O in various portions of intestinal villi during different experimental situations of this study. Mean values \pm S.E.

	Number of observations	From 5 to 30 per cent of villous length	From 5 to 50 per cent of villous length	Whole villi
<i>J jejunum</i>				
Krebs-glucose resting blood flow	14	774 \pm 23	654 \pm 20	563 \pm 15
Krebs-glucose, vasodilatation	1	570 \pm 1	514 \pm 21	473 \pm 3
Krebs-glucose, ischemia	19	471 \pm 11	440 \pm 11	411 \pm 9
Krebs-mannitol	10	711 \pm 35	629 \pm 77	550 \pm 19
Choline-mannitol	10	378 \pm 4	360 \pm 18	344 \pm 14
<i>Ileum</i>				
Krebs-glucose	14	743 \pm 32	634 \pm 4	550 \pm 16
Krebs-mannitol	9	769 \pm 48	674 \pm 37	569 \pm 14
Choline mannitol	11	351 \pm 16	343 \pm 13	337 \pm 11

and the ileum, respectively. In this situation the intestinal segments become secretory organs.

The villous circulation of blood was also altered in two ways. In one series of experiments the total venous outflow from the intestinal segments was increased from a resting value of 28 ± 2 ml (min \cdot 100 g) ($n=7$) to 147 ± 16 ml (min \cdot 100 g) by means of close arterial infusions of isopropyl noradrenaline. In this situation the mean transit time for plasma through the villous vascular loops simultaneously decreases from 4–6 sec at "rest" to 1 sec or less during vasodilatation (Biber *et al.* 1973). Concomitantly the hyperosmolality at the villous tip was reduced to 709 ± 33 mOsm/kg H₂O ($n=12$).

In another series of expts. the intestinal circulation was completely stopped by clamping the vascular supply. Prior to the vascular occlusion these segments were exposed to a choline-mannitol solution for 30 min. During the ischemia the intestinal segments were exposed to a Krebs-glucose solution that was continuously equilibrated with a gas mixture containing 5% CO₂ in O₂. Thus, the intestinal segments were in this situation metabolically as during *in vitro* conditions. The tip osmolality value was reduced as compared both to the values observed during "resting" and increased blood flow (Fig. 7).

Table I summarizes the measured mean osmolalities in different portions of the villi during the experimental series of Fig. 5–7.

Discussion

A prerequisite for determining osmolality in biological tissues by cryoscopy is a rapid freezing of the tissue. The freezing process in a salt solution always starts with a crystallization of water and a concentration of the remaining solutes until the entire solution is solidified at the so called eutectic temperature. This temperature is characteristic for each solute and has for NaCl been determined to -21.8 °C (Meryman 1966). A too slow freezing of the tissue may induce artefacts such as diffusion within an unfixed tissue and/or an extensive "salting out" phenomenon. With the present freezing method employed

TISSUE OSMOLALITY
mOsm/kg H₂O

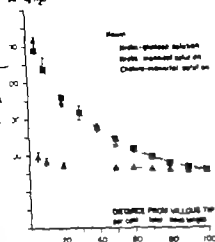


Fig. 6

TISSUE OSMOLALITY
mOsm/kg H₂O

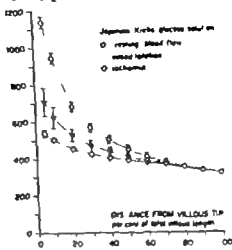


Fig. 7

Fig. 6 Osmolality in the ileocecal tissue in distal segments. After exposing the intestinal lumen to 3 different osmotic solutions. Isotonic blood flow was within the "normal" range. During perfusion with the isotonic-mannitol solution the distal segments secreted fluid. Bars indicate S.E. Number of observations, see Table I.

Fig. 7 Tissue osmolality in the ileocecal tissue in distal segments during three different circulatory conditions. Isotonic blood flow was within the "normal" range. During perfusion with the isotonic-mannitol solution the distal segments secreted fluid. Bars indicate S.E. Number of observations, see Table I.

eutectic temperature for NaCl was probably reached within 2-4 s in the villi after the lumping of intestinal vessels, which strongly indicates that such artefacts were avoided in this study.

During thawing at temperatures above the eutectic temperature, concentrated salt solutions in liquid state will surround not yet melted ice crystals. When the frozen tissue is warmed slowly as in the present study diffusion of solutes and water may take place and reduce osmotic gradients. Hence, conclusions concerning osmotic differences *between* each villous cross-section are not considered possible. The values for tissue osmolality reported in this study are essentially representative for the villous core. The osmolality of epithelial cells is probably lower than that of the villous core but could not be determined with the method used.

The results of this study are in full agreement with the countercurrent multiplication hypothesis earlier proposed by Jodal *et al.* (Haljam  *et al.* 1973, Jodal 1973, 1974). Thus, the presence of a marked hyperosmolar region was demonstrated in the upper parts of the villi reaching values above 1000 mOsm/kg H₂O. Furthermore, an osmolality gradient existed within the villous tissue, the tip osmolality being highest. These results fully agree with the observations made by Haljam  *et al.* (1973) when studying the sodium distribution in the ileocecal tissue. Changing the rate of sodium absorption in the intestinal epithelium by addition or removal of luminal glucose in the jejunum produced the expected effects (Fig. 6), i.e. increases of sodium transport by adding glucose to the luminal perfusate.

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TABLE I Mean tissue osmolality in mOsm/kg H₂O in various portions of intestinal villi during different experimental situations of this study. Mean values \pm S.E.

	Number of observations	From 5 to 30 per cent of villous length	From 5 to 90 per cent of villous length	Whole villi
<i>Jejunum</i>				
Krebs-glucose, resting blood flow	14	774 \pm 23	654 \pm 20	563 \pm 15
Krebs-glucose, vasodilatation	12	570 \pm 21	514 \pm 21	473 \pm 20
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MA 11

assumes constant in all models in Fig. 8

Extracellular sodium concentration (N_{Na}) and extracellular potassium concentration (K_{K}) at villous tip equalled 190 mmol/l and 4.0 mmol/l, respectively (Kjellner 1963). K_{K} at villous base was set to 12 mmol/l and decreased along villous length in proportion to the measured osmolality.

The extracellular space equalled 22% at the villous base (Espouzo et al. 1972). Extracellular sodium and chloride concentrations are equal.

model assumptions

Model A	Model B	Model C
<p>Constant villous extracellular space equalled 22 per cent (Espouzo et al. 1972). Sodium chloride contributed to extracellular osmolality throughout the villous in the same relative extent as at the villous base, here osmolality is set to 312 mOsm/kg H_2O.</p> <p>Activity coefficient for sodium chloride 0.92</p>	<p>1 See assumption 2 in model A.</p> <p>2 Constant N_{Na}, K_{K} ratio along the villus.</p> <p>3 Villous extracellular space calculated from other assumptions and varying between 22 and 30-35 per cent at base and tip, respectively.</p>	<p>1 See assumption 1 in model A.</p> <p>2 One-to-one exchange of sodium and potassium at base-lateral cell membrane.</p>

models it is assumed that extracellular sodium contributes to extracellular osmolality to a large extent at the base and at the tip of the villus. Model B mimicks the situation where water moves out of the cells, driven by the osmotic forces created by the counter current multiplier. In model C, the extravascular space was considered to be constant and as further assumed that a one-to-one sodium-potassium exchange occurred at the cell membrane, since the NaK-ATP-ase driven sodium pump is supposed to function in this way (see e.g. Glynn and Carlish 1975 Rose 1976).

However none of the proposed models is likely to represent the whole truth, since countercurrent multiplication is such a complex mechanism that, for example, models B and C may well exist simultaneously. Moreover the diffusion of potassium and sodium along their concentration gradients across the cell membrane has not been evaluated in any of the models. It is therefore not surprising that certain "internal" inconsistencies exist in the models. For example the intracellular osmolality seems to be higher than the extracellular one particularly in model C. However one tentative conclusion may be drawn from all these models, namely that the intracellular sodium concentration in the epithelium at the tip of the villus is high (100 mmol/l) which may be of great functional significance for absorption of glucose and amino acids (see below). Such an increase of intracellular sodium concentration is corroborated by some recent findings in the countercurrent system of the rat renal papilla (Morgan 1977).

There are many functional implications of the intestinal countercurrent multiplier some of which will be summarized here. The villous tips represent the parts of the villi that are in the most efficient contact with the luminal contents. When perfusing the intestinal lumen with an isotonic Krebs-glucose solution, the osmolality of the villous tips reached values of about 100 mOsm/kg H_2O creating a gradient of tissue to lumen osmolality of about 100 mOsm/kg H_2O which represents an enormous pressure difference. If a solute can exert

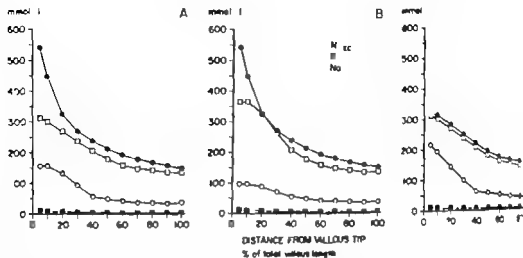


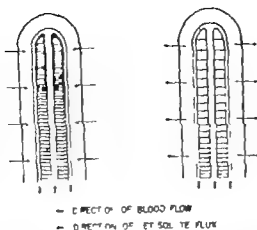
Fig. 8 Extracellular sodium concentration (Na_{ec}), extracellular potassium concentration (K_{ec}), intracellular sodium concentration (Na_{ic}) and intracellular potassium concentration (K_{ic}) in the villi calculated from the results obtained in this study and the results reported by Haljamäe *et al.* (1973). The 3 panels are based on different assumptions described in detail in Table II.

augments tissue osmolality particularly in the villous tips. Again, similar results were obtained on the jejunum when investigating the distribution of sodium in the villi (Haljamäe *et al.* 1973). Substituting all sodium ions in the luminal perfusate with choline abolished almost completely the tissue hyperosmolality (Fig. 5 and 6), strongly suggesting that countercurrent multiplication mainly involves sodium.

The efficiency of a countercurrent exchanger or multiplier is also dependent on certain vascular parameters such as mean transit time in the villous vascular loops. This parameter was altered in two ways. In one type of experiments vasodilatation was produced by propyl-noradrenaline. This lowered villous osmolality markedly (Fig. 7) according to the countercurrent hypothesis due to shortened mean transit time in the villi which reduced time for intervacular diffusion of electrolytes and water in the villous vascular loops. Similar results were obtained by Haljamäe *et al.* (1973) when studying sodium distribution in the villi. It might be argued that isopropyl-noradrenaline, a β -adrenergic agonist, may produce direct effects at the cellular level. However, *in vitro* experiments suggest that isopropyl-noradrenaline has little or no effect on electrolyte transport in the intestine (Field and McCall 1971).

In another type of experiments, intestinal blood flow was completely stopped by occluding the supplying vessels. Tissue osmolality became decreased also in this experimental situation, as compared to the tissue osmolality gradient present during "resting" blood flow conditions and during induced vasodilatation.

Combining the present results with the values for tissue content of sodium and potassium reported by Haljamäe *et al.* (1973) allows for an estimation of intra- and extracellular sodium and potassium concentrations if certain assumptions are made. Three such attempts have been pursued in Fig. 8. The various assumptions made in the different models are summarized in Table II. Briefly, in model A the extracellular space has been considered to be constant along the villus, while in model B it is supposed to vary keeping the intracellular sodium and potassium concentration ratio constant. Furthermore, in these



4 Schematic illustration of proposed mechanisms by which villus and villous structures may actively absorb or secrete fluid. In the left panel fluid absorption is accomplished by countercurrent multiplication of hypertonicity created by net solute flux into the subepithelial vessels. The resulting hypertonicity towards the tip of the villus or villous structure is indicated by the increased number of horizontal lines. In the right panel secretion of fluid is produced by creating a hypotonic compartment (indicated by horizontal lines) at the tip by countercurrent multiplication of hypotonicity. This is created by net solute flux from the subepithelial capillaries into the surroundings. The mechanism proposed is accompanied by osmolarity changes also. The blood flow there are not indicated in the Fig. Note that direction of blood flow may be reversed to that illustrated in the Fig. without any functional consequences for the proposed mechanisms.

observed *in vitro* and one may question the relevance of such *in vitro* studies for the situation *in vivo*.

The tissue hyperosmolality is probably not only of importance for transepithelial water transport but also for the movement of water *within* the villus. According to Curran's model, interstitial tissue pressure is increased in the hyperosmolar compartment. Hence, the osmolarity gradient along the villous length is probably accompanied by a corresponding hydraulic gradient which establishes a pressure head for the flow of lymph in the central vein. This explains why such a high percentage of the absorbed water (up to 50%) seems to be absorbed via the lymphatics (Simmonds 1954; Barrowman and Roberts 1967; Jodal *et al.* 1975).

According to current concepts the absorption of glucose and amino acids across the luminal membrane of the enterocyte is in part driven by a sodium concentration gradient between lumen and cytoplasm (see e.g. Crane 1962). This gradient is maintained by the sodium pumps at the baso-lateral plasma membranes of the intestinal cell. The intracellular sodium concentration (Na_{ic}) is then assumed to be fairly low. Esposto *et al.* (1973), for example, proposed that Na_{ic} *in vivo* is about 20 mmol/l. The three models discussed above infer that Na_o is at least 100 mmol/l. In fact, two of the models (A and C) implied that sodium gradients at all exist between lumen and cell. It should be pointed out, however, that the calculated intracellular sodium concentrations may represent mean values. One may imagine an uneven sodium concentration within the cell, the concentration being higher at the basal than at the luminal membrane due to the large osmotic pressure present in

its full osmotic force 800 mOsm/kg H_2O would equal about 20 atm, or around 150 mm Hg. Since most of the hyperosmolality in the villi is probably made up by sodium chloride, one has to know the reflection coefficient (σ) of this solute with regard to intestinal epithellum to be able to evaluate the magnitude of the osmotic forces involved. Fordtran *et al* (1965) made an attempt to estimate σ for NaCl in the human small bowel. They arrived at values of 0.58 and 0.95 in the jejunum and ileum respectively. There are, however, theoretical difficulties in determining σ for NaCl in the intestine since sodium is actively transported by the enterocytes. Furthermore, ions are charged and the "walls" of the intestinal pores are considered to be electrically negative. Using a value of 0.6 as a rough estimate of the intestinal σ for sodium chloride it can be calculated that an osmotic pressure head of about 9 000 mm Hg would be present across the epithelial lining at a villous tip. In the upper third of a cat villus exposed to a Krebs-glucose solution it can be similarly calculated that an average osmotic pressure head of about 5 000 mm Hg exists (see Table I).

Such large pressure differences must be of great importance for water absorption. Therefore, in agreement with Curran's model for water absorption it is proposed that the villous hyperosmolar region, demonstrated in the present study, represents the hyperosmolar compartment necessary for explaining intestinal water absorption *in vivo*. This conclusion is corroborated by the present observation that marked reductions of intestinal water absorption and hyperosmolality, e.g. by luminal perfusion with choline mannitol solutions (Fig. 5 and 6), turned the intestine into a secretory organ. This observation may, in turn, be of importance in explaining certain types of diarrhea. A more detailed experimental analysis of the relationship between water absorption and villous tissue osmolality will be presented in subsequent studies.

The magnitude of the osmotic forces involved *in vivo* during water absorption may imply that solvent drag plays a significant role in epithelial transport in the gut. This, in turn, has at least two important functional implications. — *First* according to Dornier and Bossert (1967) the intercellular spaces constitute the site of "standing osmotic gradients" proposed to be of great importance for water absorption. This hypothesis was originally proposed assuming that the luminal end of intercellular spaces was completely "tight". However, recent experimental evidence strongly indicates that this is not the case in some epithelia including the gut (Frömter and Diamond 1972). Since the flow of fluid through these paracellular spaces must be very high, indeed it is questionable if any gradients of the type proposed by Diamond really exist *in vivo* in the intestinal epithellum. Furthermore, a high concentration of extracellular sodium chloride is created at the "tissue end" of the intercellular space by the countercurrent multiplier which, in turn, tends to create an intercellular concentration gradient of opposite direction to the one proposed by Diamond. — *Second* the presence of large unstirred layers in the intestinal lumen has been proposed by e.g. Dietschy (1975) and these layers are believed to be of great functional significance, e.g. lipid absorption. However, such studies have been largely performed in *in vitro* conditions where increases in tissue osmolality created by "active" mechanisms are probably only a fraction of those present *in vivo* (cf. ischemia, Fig. 7). Consequently the extent of diffusion and convective stirring in the lumen must quantitatively be much less

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the villous tissue, forcing water out of the cells at their basal end. This suggestion is corroborated by the uneven intracellular potassium concentration reported by Zentgraf & Monge (1975). Moreover the luminal concentration of sodium adjacent to the cells is higher than that of perfusate. This is suggested by the somewhat higher osmolality in the lumen close to the cells (see Results). It is, however, difficult to determine this osmolality partly because of the different calibration curves for tissue and electrolyte solutions (see Fig. 2).

In textbooks of physiology it is usually proposed that villous structures, like those present in the gut, are primarily designed to increase surface area in order to enhance the transport capacity of various solutes (cf. Wilson 1962). Most villous and papillary structures are, however, supplied with a vascular arrangement similar to that of the villi in the small intestine, implying the presence of a countercurrent exchanger. It is therefore proposed that villous and papillary structures in many organs are designed mainly to act as countercurrent multipliers, thereby primarily enhancing water transport. One may even have villi that "secrete" fluid due to a countercurrent multiplication of a hypotonicity (see Fig. 1) when net solute flux is directed from plasma to lumen. These proposals are substantiated by recent experimental findings that the filiform and fungiform papillae on the cat's tongue exhibit an osmolality gradient of the same type as in intestinal villi, tip osmolality being 800–950 mOsm/kg H₂O when exposed to a Krebs-glucose solution. As in the small intestine this osmolality gradient is decreased by exposing the tongue to a choline-mannitol solution (observations to be published). Admittedly the papillae of the tongue are not important for overall water absorption but the presence of a hyperosmolar tissue region along these villi-like structures strongly suggests that the papillae of the tongue are supplied with the vascular arrangement and the "active" transport mechanisms necessary for an efficient countercurrent multiplication.

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An "on-line" colorimetric method for repeated, rapid determinations of capillary diffusion capacity

By

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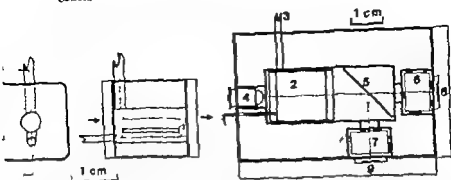
Abstract

RIPPE, B. and L. STAGE. An "on-line" colorimetric method for repeated, rapid determinations of capillary diffusion capacity. *Acta physiol. scand.* 1978. 102. 108-115.

The single injection indicator diffusion method for estimation of capillary diffusion capacity (Crone 1963) has been developed to provide directly visualized, continuous colorimetric recordings of the venous concentration curves by means of a two-colour densitometer system. Cardio-Green, bound to albumin, is used as the non-permeant ("reference") tracer and Cr-EDTA as the permeant one. The artificially perfused hindquarter muscle vascular bed of rats is used. Highly reproducible curve recordings can be obtained every fourth minute. Accidental disturbances of the recordings are readily detected. Thus, compared to fractional venous sampling technique, the present technique has the great advantage of allowing immediate, controlled and frequently repeated determinations of capillary diffusion capacity. It is therefore easily combined together with measurements of filtration-absorption events, e.g. for frequent quantitative comparison of capillary diffusion and filtration capacities over a wide range of induced changes in perfused capillary surface area and/or capillary permeability. The main limitation is that the employed permeant tracer necessitates the use of erythrocyte-free perfusates.

The single injection indicator diffusion method (Chinard *et al.* 1955; Crone 1963) is frequently employed for investigations of the transcapillary diffusion exchange. The applicability and validity of this method has been discussed extensively earlier (*cf.* Crone and Lassen 1970; Lassen and Trap-Jensen 1970; Bassingthwaite 1974). Until now the venous concentration curves have been obtained by fractional venous sampling, implying that numerous samples must be collected and subsequently determined with respect to concentrations, to allow for a single analysis of capillary diffusion exchange. As this is a fairly laborious procedure it is seldom possible to obtain more than a few such analyses in an individual experiment, and for essentially the same reason there have been difficulties in combining the single injection technique with other techniques for functional capillary studies.

On this background the single injection technique was modified to make possible a direct visualization of the venous time-concentration curves, and thus allow for frequently repeated determinations that could be immediately checked. One of the main reasons was to facilitate the use of the method for quantitative comparisons of capillary diffusion and filtration ex-



Left part shows the cuvette part of the densitometer. The broken line at right shows the flow path through the large vessel. The light path. Right part shows the densitometer 1 inlet, 2 cuvette, 3 outlet, 4, 5 double glass wall, 6 542 nm filter, 7 805 nm filter 8 and 9 photocells. The broken line shows the path.

injected intravascular tracer injections were performed by means of a precision step dispenser delivering a constant amount of injectate in every bolus ($40 \mu\text{l} \pm 0.4 \mu\text{l}$ mean \pm S.D.). It means that with every injection 6-8 moles of CG-albumin and 3-4 micro moles of EDTA entered the vascular bed. The injectate was delivered to the perfusate inflow minute mixing chamber that efficiently mixed the injectate with the perfusate.

pH measurements. pH of the venous effluent was measured continuously to correct for pH dependence of the permeant tracer curve. A pair of ordinary pH electrodes placed the effluent from the densitometer cuvette were used. pH was recorded on one channel of a potentiometer writer via an amplifier unit.

The pH recording channel was also used to mark the start and end of each tracer injection means of an automatic switch in the step dispenser coupled to the pH amplifier unit. It was performed to allow measurements of mean transit times.

Recording devices and curve recordings. The small recording cuvette (volume about 3 ml) is shown in Fig. 2 left part, and the cuvette-densitometer unit in the right part of Fig. 2. In the cuvette light passed through 16 mm of the perfusate. It is of great importance that the contents is well stirred. This was achieved by an arrangement inside the cuvette that allowed minor recirculation and hence a stirring effect, where the inlet provides the driving force. The outflow resistance was moderately increased by the stirring device which, however, in the experimental situation was compensated for by keeping the venous outflow pressure up lowered to avoid undue increases in capillary pressure.

In the right part of Fig. 2 the mirror 5 is a double glass wall which splits the light beam into two filters (6 for Cr-EDTA and 7 for CO), which are of the interference type with peak transmissions at 542 and 805 nm, respectively (DAL, ZEISS West Germany). Non-linearity in the CO recordings may occur with some CO light filters. The reason for this is a light difference between the CG absorption peak and the peak of transmission of the light filter.

Photocells 8 and 9 are of type BPW33 (Siemens) and their outputs are connected to two amplifiers as shown in Fig. 3. The input part to the left acts as a current-to-voltage amplifier. The subsequent part is a balanced logarithmic amplifier with two BC239 acting as log-

For such purposes the preparation was continuously weighed, and arterial pressure (in a artery) and venous pressure (in sidebranch of caval vein) could be set to any desired level. In addition flow could be varied by adjusting the setting of the perfusion pump. Venous outflow was repeatedly collected directly in a graduate cylinder tube to check the setting of the pump. Flow was always kept above 11 ml/min \cdot 100 g muscle tissue to eliminate the risk for significant flow limitation of transport. This was checked by repeated determinations of diffusion capacity over a wide range of flows. Flow dependence of diffusion capacity was only evident at flows below 10 ml/min \cdot 100 g of tissue.

This *in vitro* preparation has the great advantage that it can be utilized both for studies during maximal vasodilatation, allowing analyses of capillary events during perfusion of the entire capillary surface at normal or altered permeability and during exactly graded vasoconstrictions, or vascular microplugging, implying alterations also of the perfused capillary surface area. For direct quantitative comparisons of diffusion and filtration events it is obviously of great importance that essentially all other relevant hemodynamic parameters can be closely controlled.

3 Properties of the tracer molecules When bound to albumin CG (m.w. 775) has a strong and narrow absorption peak at 805 nm and practically none at 542 nm, where Cr EDTA has its strongest absorption. One disadvantage is that diluted CG is relatively unstable, but its absorption decreases only 2-3 per cent during the few hours needed for the experiment, which can easily be corrected for.

Cr EDTA (m.w. 341) has its strongest light absorption at 542 nm and almost no absorption at 805 nm. Its light absorption at 542 nm is somewhat dependent of pH. However in the pH-range of use (6.6-7.4) correction can easily be made since the absorption here decreases by about 2% per 0.1 unit of pH increase.

In this type of colorimetric recording, Cr EDTA is given in considerably higher concentrations than when radioactive Cr EDTA is used. To exclude significant admixture of free chromium ions or free EDTA in the injectate, which might have toxic effects respectively bind calcium ions, the following procedures were used. 0.2 molar solutions of CrCl₃ and Na₂H EDTA were mixed with CrCl₃ in slight excess. The mixture was boiled, allowed to cool and pH was adjusted to 7.3 with NaOH. The solution was left for 24 h to allow excess chromium to precipitate as chromium hydroxide and was then filtered. It could be calculated that a 0.1 mol/l Cr EDTA solution, produced according to these principles, has a Cr³⁺ concentration as low as about 10^{-6} mol/l and "free" EDTA about 10^{-5} mol/l. Since each tracer injection contained only 3-4 micro mol Cr EDTA the amounts of Cr³⁺ and free EDTA that reached the tissues were negligible.

4 Tracer injections. The injectate contained 70-100 mmol/l Cr EDTA \pm 0.3 mmol/l CG 6-10 mmol/l albumin (Human Serum Albumin, Kabi, Sweden) and about 250 mmol/l NaCl. The albumin content eliminated the possibility that free CG could reach the preparation.

The injectate was hyperosmolar (6-800 mosmoles/l) mainly as a result of the way Cr EDTA was prepared (see above). However because of the minute amounts injected (40 μ l) in relation to flow (>10 ml/min) no detectable osmotic transient was created, as judged from the continuous weight recordings.

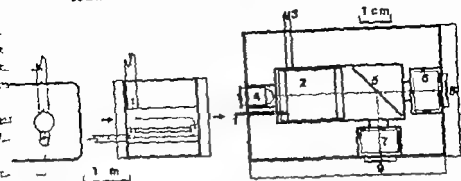


Fig. 2. Left part shows the cuvette part of the densitometer. The broken line at right shows the flow path of the large arrows (the light path). Right part shows the densitometer: 1. inlet, 2. cuvette, 3. outlet, 4. stop, 5. double glass wall, 6. 542 nm filter, 7. 805 nm filter, 8 and 9. photocells. The broken line shows the light path.

Sudden intraarterial tracer injections were performed by means of a precision step dispenser delivering a constant amount of injectate in every bolus ($40 \mu\text{l} \pm 0.4 \mu\text{l}$, mean \pm S.D.), by means that with every injection 6-8 μ moles of CG-albumin and 3-4 μ moles of Ca^{++} -EDTA entered the arterial bed. The injectate was delivered to the perfusate inflow minute mixing chamber that efficiently mixed the injectate with the perfusate.

pH measurements. pH of the venous effluent was measured continuously to correct for the pH dependence of the permanent tracer curve. A pair of ordinary pH electrodes placed in the effluent from the densitometer cuvette were used. pH was recorded on one channel of a potentiometer writer in an amplifier unit.

The pH recording channel was also used to mark the start and end of each tracer injection by means of an automatic switch in the step dispenser coupled to the pH amplifier unit. This was performed to allow measurements of mean transit times.

6. Recording devices and error minimization. The small recording cuvette (volume about 3 ml) is shown in Fig. 2 left part, and the cuvette-densitometer unit in the right part of Fig. 2.1. The cuvette light passed through 16 mm of the perfusate. It is of great importance that the content is well mixed. This was achieved by an arrangement inside the cuvette that allowed a minor recirculation and hence a stirring effect, where the inlet provides the driving force. The outflow resistance was moderately increased by the stirring device which, however, in the experimental situation was compensated for by keeping the venous outflow pressure lowered to a value which increases in capillary pressure.

In the right part of Fig. 2 the mirror 5 is a double glass wall which splits the light beam into two filters (6 for Ca^{++} -EDTA and 7 for CG), which are of the interference type with narrow transmissions at 542 and 805 nm, respectively (DAL, ZEISS West Germany). Non-linearity in the CG recordings may occur with some CG light filters. The reason for this is the light difference between the CG absorption peak and the peak of transmission of the light filter.

Photocells 8 and 9 are of type BPW33 (Siemens) and their outputs are connected to two amplifiers as shown in Fig. 3. The input part to the left acts as a current-to-voltage amplifier and the subsequent part is a balanced logarithmic amplifier with an MC239 acting as log-

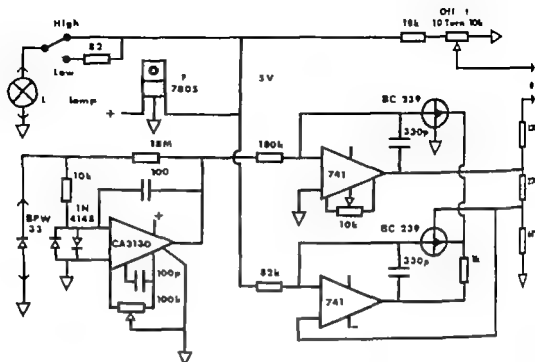


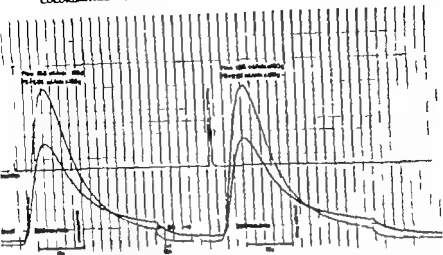
Fig. 3 Schematic diagram of the photocell amplifier. The amplifier CA3130 is used as current-to-voltage amplifier and the two 741 form a logarithmic amplifier. F7805 is a voltage regulator.

arithmetic elements. The transistors have to be thermally tied together. The outputs were recorded on two channels in a three-channel potentiometer recorder (Rika Denki, model B34). Full scale deflection time < 1 s. Actual recordings of venous time-concentration curves are shown in Fig. 4.

Calibration of the densitometer measuring system was performed by aspirations of reference samples, containing known concentrations of the injectate, through the circuit system, where the pH measuring device automatically gives the pH of the solutions.

7 Analysis by calculator. The analysis of the tracer curves was made in a calculator system (Hewlett Packard 9820A). The curves were sampled and entered into the calculator via a digitizer unit (HP 9864A). In the first computation step the possible nonlinearity of the recording, as well as the pH dependence of the Cr-EDTA recording, was corrected for. The curves were standardized in relation to the calibration recordings, mentioned above. The traced curves were then plotted by a calculator plotter in a normalized form with respect to peak CG concentration (Fig. 5).

In the subsequent computation step areas under the tracer curves were estimated in order to obtain approximate values for mean transit time and flow. Since the single injection curve for diffusion estimations were usually registered for only the first minute, estimations of mean transit time and flow necessitated that the curve tails were either extrapolated, or the entire curve recordings were performed. The entire curve recordings showed that the tracer concentrations did generally not reach zero levels until after 3–4 min, and further a logarithmic plotting revealed that both the permeant and the non-permeant indicator curve tails were multiexponential (Fig. 5).



4. Original recording of two consecutive venous time-concentration curves during constant hemodynamic conditions. In this case the injectate calibration value for Cr-albumin equalled that for Cr-EDTA. pH recording for the venous effluent is also shown, with the superimposed injection markings.

the apparent fractional extraction function, $E(t)$, is defined as:

$$E(t) = \frac{C_n(t) - C_p(t)}{C_n(t)}$$

where C_n is the standardized concentration of the non-permeant tracer and C_p that of the permeant one at time t , and $E(t)$ was computed and plotted (Fig. 5). E varies with time, only because of nonuniformity in design of capillary network and in flow interarteriolar and longitudinal diffusion between the permeant and non-permeant tracer and back diffusion (cf. Crone and Lassen 1970). To get a weighted mean of the extraction occurring at all exchange vessels, i.e. the "short" ones with low extraction as well as the "long" ones with high extraction, the overall extraction was calculated according to the formula

$$E(T) = \frac{\int_0^T C_n(t) dt - \int_0^T C_p(t) dt}{\int_0^T C_n(t) dt}$$

E calculated in this way, i.e. by means of the "area method" was plotted as a function of different integration end points (T -values) and formed a plateau at T -values between peak concentration time of the tracer curves and the time where they had declined to about 50% of peak value (Fig. 5).

This plateau value for E was used in the calculations of the capillary diffusion capacity or PS-product (Renkin 1959) according to the expression $PS = Q_p \ln(1/E)$ where Q_p is the directly measured perfusate flow assuming monoexponential concentration profile in the exchange vessels.

8. *Experimental errors.* When all hemodynamic parameters were kept constant the random

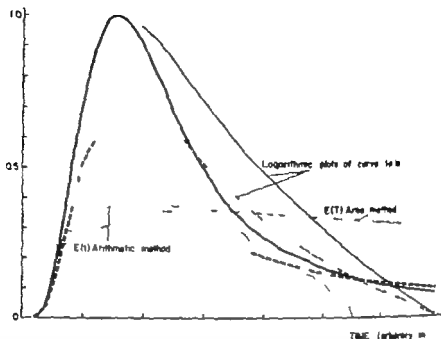


Fig. 5 Computer diagram for the tracer curves shown to the right in Fig. 4. The curves are here standardized to the injectate calibration and plotted in a normalized form with respect to peak CG concentration. The curve tails are also plotted logarithmically. Not their multiexponential decay. In addition, the "area" fractional extraction $E(t)$, as well as the "integrated (real) extraction $E(T)$ are shown. The following computed data were obtained from the original recordings. $E(T)$ (area method): plateau-time 8.4 s, $PS = 5.92$ ml/min/100 g, flow computed from the CG curve = 14.58 ml/min/100 g (directly measured flow = 13.50 ml/min/100 g). Mean transit time for CG = 15.6 s and for Cr EDTA 2.8 s. Flow and transit times were here estimated after monoexponential extrapolation from the portion where the CG curve had decreased to 10 per cent of its peak concentration.

experimental error determined as the coefficient of variation between consecutive PS determinations in 25 expts. was less than 3 per cent in a given expt (cf Fig. 4). Variations between individual preparations, was of the order of 10 per cent, which includes experimental error and biological variation.

As regards estimation of flow and mean transit times from the curves, systemic errors were induced by using monoexponential extrapolation of the multiexponential curve (cf Fig. 5). If however the extrapolation was performed after the curves had declined to 10% of peak value, computation of flow from the CG curve gave a systemic overestimation of this parameter of only 5–10%. The error becomes much greater if mean transit time is calculated from the extrapolated curve instead of from the total curve, registered for 3–4 min, simply because the end of the tails can be quite long as a result of the multiexponential decay. Therefore, in circumstances where mean transit times are to be used for experimental purposes, it is advisable to record the total curve, i.e. for 3–4 min.

The presence of a multiexponential decay is not surprising since even a fairly "uniform" skeletal muscle contains portions (e.g. "tonic" and "phasic" muscles) that differ somewhat in their vascular arrangements both in terms of flow conductance and capillary exchange area. Further, in some parts of the preparation, circulation may have been somewhat disturbed due to tissue ligatures, etc.

Comments

The main advantage of this modification of the indicator diffusion method is that a great number of recordings can be frequently performed with high precision and reproducibility and under direct visual control. Consequently accidental interferences can in most cases be immediately detected from the curve characteristics. It further allows repeated recordings before, during and after induced, changes of the hemodynamic situation, whether brought about "passively" by changes in pressures and flows or by "active" changes in vascular smooth muscle activity and/or in endothelial permeability. Furthermore, the recordings of diffusional transport can readily be associated with concomitant determinations of the filtration-absorption and of the hydraulic conductivity in the capillary section (CFC measurements). Series of such experimental studies have been performed and will be presented in subsequent publications. Lastly it might also be possible to use diffusible tracers of different molecular weights, for repeated estimations of restricted diffusion effects.

The method has one obvious drawback: the use of Cr-EDTA as the diffusible tracer for colorimetric recordings makes it necessary to use cell-free perfusates. It might, however, not be impossible to find a suitable diffusible indicator which, like the non-permeant CG 500, can be used also during ordinary blood perfusion. This problem has not been approached so far since the method was developed primarily to be used during some basic experimental situations, where parameters like pre- and postcapillary resistances, vascular tone, pressures, capillary surface area etc. could be quantitatively controlled, and then an *in vitro* system is preferable.

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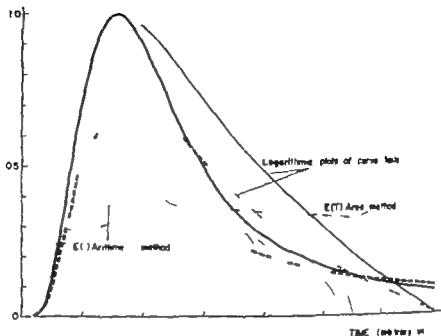


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TABLE I Dry weight (ascosone dried powder) and choline acetyltransferase activity of ventricles in untreated rats and in rats treated with chlorisondamine. Values are mean \pm S.E. Number of observations indicated in brackets.

	Dry weight mg	Enzyme activity	
		In μ g ACh/h/ventricle (total activity)	1 μ g ACh/h/g ascosone powder (concentration)
Chlorisondamine	89.7 ± 2.2 (9) ^a	16.3 ± 0.9 (9) ^a	180.6 ± 7.4 (9) ^a
control	113.2 ± 2.2 (9)	22.1 ± 0.7 (9)	193.7 ± 3.8 (9)

0.05 0.001 When the ventricles of the treated rat are compared with those of the control litter mate.

Choline acetyltransferase was determined according to the method of Catherine Hebb (see Nordenfiet 1963). Of the tissue extract 0.4 ml was incubated for 1 h at 38°C. The acetylcholine formed was extracted on to 1 mg rosin powder and expressed as μ g acetylcholine chloride formed per h per ventricle of each heart (total activity) and as μ g acetylcholine chloride formed per h per g ascosone powder (concentration); the tissue extracts from the drug treated rat and its untreated litter mate were incubated simultaneously and the reactions were assayed on the rosin rosin powder.

For comparison report on the activity of choline acetyltransferase in ventricles of rats given the β -adrenergic stimulating drug isoprenaline sulphate has been included. The ventricles are analysed in connection with study on salivary glands (Eklund 1974). Of the drug 3 mg in 0.2 ml saline was given every 8 h for 21 days. The rats were of the same sex, age, weight and strain as those given the ganglion blocking drug and further, the ventricles were treated in the method above.

For statistical analysis Student's *t*-test was used, paired comparisons were made between the treated rat and its untreated litter mate. *P*-values less than 0.05 were considered significant.

Results

Treatment with chlorisondamine At the start of the experiment no difference existed in body-weights between the rats to be given the drug and those to be used as controls. At the end of the experimental period the drug treated rats were found to have lost weight, while the weight of the control rats had remained unchanged, expressed as a percentage the body weights of the drug treated rats were (mean \pm S.E.) $82.3 \pm 1.0\%$ ($n=9$) of those of the untreated rats ($p=0.001$). The dry weights (in ascosone dried powder) of the ventricles in the drug treated rats were lower ($p=0.001$) than in the untreated ones (Table I); the weight of the muscle in the treated rats being $79.1 \pm 0.8\%$ ($n=9$) of that in the untreated rats (Fig. 1). The total activity of choline acetyltransferase in the ventricles of the treated rats was $73.4 \pm 3.0\%$ ($n=9$) of that in the controls; the difference in total activity between the two groups of animals was significant at the $p=0.001$ level. When the activity of the choline acetyltransferase as expressed in terms of concentration no significant ($0.05 \leq p < 0.1$) difference was found between treated and untreated animals.

Treatment with isoprenaline Since both the dry weight and the total activity of choline acetyltransferase of the ventricles were reduced after the treatment with chlorisondamine (resulting in the unchanged enzyme concentration), the question arose whether a change in

Fall in choline acetyltransferase activity in the ventricles of the rat heart after treatment with a ganglion blocking drug

By

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Abstract

Ekström, J. Fall in choline acetyltransferase activity in the ventricles of the rat heart after treatment with a ganglion blocking drug. *Acta physiol. scand.* 1978. 102. 116-119.

The choline acetyltransferase activity was found to be 27% lower in the ventricles of rats which had been given the ganglion blocking drug chlorisondamine than in those of untreated rats. Since the weight of the ventricles was also lower in the treated rats than in the untreated ones, it was wondered whether a decrease in the muscle mass as such affected the activity of the enzyme. Isoprenaline treatment was found to cause the muscle mass to increase markedly while it left the enzyme activity unchanged. The decrease in enzyme activity observed after the prolonged ganglion blockade is interpreted as being the consequence of a reduced traffic of impulses along the postganglionic parasympathetic nerves of the ventricles. Thus, the results of the present investigation give further support for the idea of a vagal innervation of this part of the heart.

Activity of the acetylcholine forming enzyme, choline acetyltransferase, has been demonstrated in the ventricles of the rat heart (Ekström 1970). This finding seems to support the idea of a parasympathetic innervation of the mammalian ventricles (see DeGeest et al. 1968 and Higgins, Vatner and Braunwald 1973). In a recent investigation (Ekström and Lindström 1977) the choline acetyltransferase activity was found to be reduced in the postganglionic parasympathetic nerves in the parotid glands of rats after prolonged treatment with a ganglion blocking drug. The decrease in enzyme activity is attributed to a reduced traffic of impulses along these nerves (see Ekström 1975). In the present study the effect of ganglion blockade on the choline acetyltransferase activity of the ventricles is described.

Methods

Female rats, 3-5 months old and weighing about 200 g, of a Sprague-Dawley strain bred at this institution were used. The ganglion blocking drug chlorisondamine chloride was given in 0.2 ml saline i.c. every day over a period of 13 days. The dose was gradually increased, starting with 1.5 mg per injection and ending with 3 mg; the total amount given of the drug being 81.5 mg. Untreated litter mates served as controls to the drug treated rats. The rats were killed with ether the day after the last injection. The hearts were removed and the atria separated from the ventricles. Acetone dried powder was then obtained from the ventricles. The powder was made up in cysteine saline in a concentration of 50 mg/g wet weight.

Discussion

The present study shows that prolonged treatment with a ganglion blocking drug lowers the activity of choline acetyltransferase in the ventricles of the rat heart. It is of interest to note that the reduction in enzyme activity observed was of the same magnitude, expressed as percentage, as that found in the parotid glands of these animals (Ekström and Lindmark 1977). As pointed out by these authors the fall in the choline acetyltransferase activity of the preganglionic nerves of the parotids caused by the drug was probably a specific effect and not due to a general toxic action on cholinergic nerves, since the activity of this enzyme was not reduced in the preganglionic sympathetic nerves of the adrenals (Ekström and Lindmark 1977). After the treatment with the ganglion blocking drug the weight of the ventricles was found to be lower than in untreated animals. The percentage fall in ventricular muscle weight was of about the same magnitude as that in body-weights. The outcome of the expt. using oprenaline indicates that a change in the muscle mass as such is not accompanied by a similar change in choline acetyltransferase activity—this is an observation in agreement with studies on skeletal muscles (Diamond, Franklin and Milley 1974) and salivary glands (Ekström 1974).

It seems reasonable to interpret the decrease in choline acetyltransferase activity in the ventricles which was found after the treatment with the ganglion blocking drug as being a consequence of a reduced flow of impulses in the postganglionic parasympathetic nerves. This was also the suggested explanation of the fall in the enzyme activity in the parotid glands (Ekström and Lindmark 1977). Thus, the results of the present investigation seem to give further support for the concept of a vagal innervation of the ventricles of the heart.

This work was supported by grants from the Faculty of Medicine in Lund. Chlorpromazine was kindly supplied by Hälsjö-Ciba-Geigy AB, Sweden.

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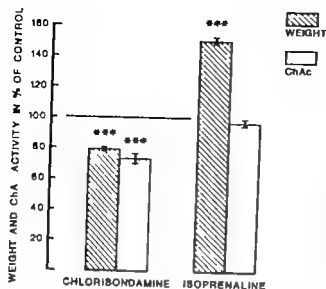


Fig. 1 Dry weight and choline acetyltransferase activity per ventricles of treated rats either with chlorisondamine or isoprenaline and of their corresponding litter mates. The weight and the enzyme activity are expressed as percentage of those of the ventricles of the control litter mates (mean \pm S.E.). Most of comparisons in the experiment with chlorisondamine is 9 and in the experiment with isoprenaline 6. *** indicates a P value less than 0.001.

the ventricular mass in itself would affect the total choline acetyltransferase activity. Prolonged treatment with isoprenaline has been found to cause the ventricular weight of the heart to increase (Schneyer 1962) a finding which was confirmed (unpublished) in connection with a study on salivary glands (Ekström 1974). The effect of this drug on the weight and choline acetyltransferase activity of the ventricles is now reported.

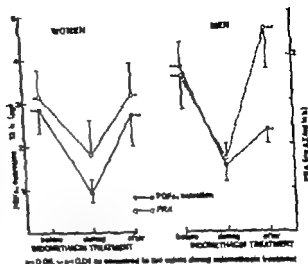
Neither at the start nor at the end of the experimental period was there any significant difference in body weights between the animals given the drug and their untreated litter mates. The dry weights of the ventricles were markedly increased ($p < 0.001$) after the treatment as a percentage the ventricles of the isoprenaline treated rats weighed 149.6 ± 2.4 ($n = 6$) of those of the controls (Table II, Fig. 1). The total activity of choline acetyltransferase in the ventricles of the treated rats was about the same as that in the ventricles of the animals serving as controls. In percent it was 96.7 ± 1.6 ($n = 6$) when ventricles of the treated rats were compared with those of the untreated ones. Consequently the concentration of choline acetyltransferase was lower ($p < 0.001$) in the ventricles of the treated rats than in those of the untreated rats.

TABLE II Dry weight (acetone dried powder) and choline acetyltransferase activity of ventricles in treated rats and in rats treated with isoprenaline. Values are mean \pm S.E. Number of observations is indicated in brackets.

	Dry weight mg	Enzyme activity	
		I / g ACh/h/ventricles (total activity)	In μ g ACh/h/g acetone powder (concentration)
Isoprenaline	191.9 ± 6.3 (6) ^a	23.4 ± 1.9 (6) ^a	121.7 ± 7.0 (6) ^a
Control	128.3 ± 3.8 (6)	4.0 ± 1.6 (6)	188.3 ± 10.8 (6)

^a $p < 0.01$ < 0.001 When the ventricles of the treated rats are compared with those of the control litter mates.

Fig. 1 The effect of indomethacin on the prostaglandin $F_{2\alpha}$ and plasma renin activity in 9 women and 12 men with essential hypertension. The values are mean \pm S.E.



$p < 0.05$, $p < 0.01$ as compared to two points during indomethacin treatment

in women ($r = 0.532$, $p = 0.05$) and men ($r = 0.613$, $p < 0.01$). The inhibition of PG synthesis has been documented: reduce glomerular filtration rate and Na^+ excretion in some studies (Korup et al 1975; Donker et al 1976), whereas this has not been verified in all papers (Korup et al 1976). In the present study treatment of 6-7 days with the conventional indomethacin dosage did not suppress the urine volume and electrolyte excretion (Na^+ and K^+). Our results indicate that there is a close correlation between the decreases of PG excretion and PRA in patients with essential hypertension during the indomethacin treatment. In contrast to hypertensive animals (Schölkens and Siesbach 1975; Levy 1977), the rise of P in man was relatively slight. This may be explained by the fact that the decrease in the production of PGs, many of which have vasodilatory effects, is compensated by a concomitant decrease in PRA. An increase of this magnitude in BP is not of marked importance in slightly hypertensive patients. On the other hand, in patients with severe hypertension the compensatory mechanisms are further impaired, and the pressor action of prolonged treatment with anti-inflammatory analgesics might be of practical significance.

Table 1 Blood pressure before, during and after the treatment with indomethacin (75 mg daily for 6-7 days) in 9 women and 12 men with essential hypertension. The values are mean \pm S.E.

	BP	12 rats ^a before	Indomethacin treatment		
			1 day before	6-7th day during	1 week after
women	sys.	176 \pm 9	177 \pm 9	183 \pm 9	168 \pm 9 ^a
	diast.	113 \pm 3	114 \pm 3	113 \pm 3	109 \pm 2 ^a
men	sys.	163 \pm 4	158 \pm 6	156 \pm 5	154 \pm 5
	diast.	113 \pm 2	111 \pm 2	112 \pm 2	107 \pm 2 ^a

^a The latest values during the "run-in" period.

Dependence of plasma renin activity on prostaglandin excretion in essential hypertension

By

PAULI YLITALO HEIKKI VAPAATALO, TIMO METSÄ KETELÄ and TIMO PITKÄLÄINEN

Indomethacin decreases prostaglandin (PG) synthesis and plasma renin activity (PRA) in experimental animals (Schölkens and Steinbach 1975 Romero *et al* 1976) and also in man (Patak *et al* 1975 Rumpf *et al* 1975). A slight simultaneous increase in blood pressure has been found. Thus, inhibitors of PG synthetase cause a sensitization to some factors that elevate BP (Lee *et al* 1976 Negus *et al* 1976). In man, this elevation of BP has been connected to the decrease in group A of circulating PGs. However, no clear marked correlation between plasma prostaglandin A and any changes in renal function or BP could be found (Patak *et al* 1975 Lee *et al* 1976). We can now report a close correlation between the decreases in PRA and in PG urinary excretion caused by PG synthetase inhibition in both female and male patients with essential hypertension. Special attention was directed to the alteration in BP and the urinary excretion of electrolytes was also measured.

Twenty-one untreated, otherwise healthy patients (9 women and 12 men, aged from 40 to 65 years) with recently diagnosed essential hypertension were treated daily with 75 mg (25 mg a.m. and 50 mg p.m.) of indomethacin (Indetrit® Medica Ltd. Helsinki) for 6-7 days. BP was measured in supine position by a brachial cuff and a mercury manometer. The diastolic pressure was recorded at the fourth Korotkoff's sound. Before the measuring of BP the subjects rested in bed for 10 min. The mean of 3 consecutive values was taken into account. PGF_{2α} and PGE were measured by radioimmunoassay according to the method of Goldman *et al* (1972) in 12 h urine collected overnight. For the radioimmunoassay of a metabolite of PGF_{2α} (13,14-dihydro-15-keto-PGF_{2α}) commercial kits (Clinical Assays Inc., Cambridge, Mass.) were used. Urinary Na⁺ and K⁺ were determined by a flame photometer and PRA by the radioimmunoassay of A I released in the incubation as described by Fyhrquist *et al* (1976).

BP tended to increase during the indomethacin treatment. The difference was significant when BP after drug withdrawal was compared to that during the indomethacin treatment with the exception of systolic BP in males (Table I). PGF_{2α} excretion was suppressed 1/2-1/4 from the initial levels by indomethacin (Fig. 1). There was a similar decreasing tendency in PGF_{2α} metabolite and PGE excretions during indomethacin treatment. The time-concentration curve of PRA followed closely the PGF_{2α} excretion. Significant correlations between the decreases caused by indomethacin in these parameters were calculated in both

Binding of ^3H 5-hydroxytryptamine to synaptic plasma membranes of rat brain

By

J. GRIMMERO and S.-E. JANSSON

Studying the binding of neurotransmitter substances and drugs to membrane preparations from the central nervous system is a new approach to examining receptor functions. However, such studies have been performed using long incubation times, solutions lacking essential cations and with crude membrane preparations which have been stored frozen for long periods or at 0°C (Scerban *et al.* 1975, Bennett and Snyder 1976, Filloon *et al.* 1976). The use of less physiological conditions may distort agonist-receptor interactions and lead to conclusions not necessarily valid *in vivo*. Using a rapid filtration technique and short incubation times we have studied the binding of ^3H -5-hydroxytryptamine (^3H -5-HT) to synaptic plasma membranes of rat brain and compared the time course of the binding in an ionic electrolyte solution with that in a buffer solution devoid of essential cations. We report on the effect of storage of the membrane preparation and preliminary results on the saturation characteristics of the binding reaction.

aqueous-Dewey rats (200 g) were decapitated, the brains immediately removed and the cerebelli discarded. After washing in ice-cold 0.3 M sucrose supplemented with 0.5 mM EGTA, the material was homogenized according to Gray and Whistaker (1962). After centrifugation (1000 g for 5 min) and one wash by sedimentation, the pooled supernatants were spun down at 15000 g for 15 min, the pellets resuspended in 0.3 M sucrose (10 and 5 g^{-1} original tissue) and layered on top of a gradient made up of 12 ml 5 M sucrose and 11 ml 1 M Tris-HCl (Pharmacia, Sweden) in 0.32 M sucrose. After centrifugation at 21000 g for 40 min the membranes at the 5-11 interface were collected, washed with sucrose and resuspended for 15 min in 5 mM Tris-HCl buffer pH 7.4 for 40 min at 0°C with stirring. Synaptic plasma membranes were isolated from the lysate by sedimentation-flotation centrifugation according to Jones and Mayne (1974) at 40000 g for 120 min. The membrane fraction was washed with 20 mM Tris-HCl, pH 7.4 after sedimentation at 21000 g for 30 min resuspended in small volumes of Tris-HCl and used immediately or stored for 4 h at 0°C under nitrogen. The yield averaged 0.5 mg protein g^{-1} original tissue. Aliquots of the membrane suspension were added to 20 or 30 μmol Tris-HCl buffer pH 7.4 or to medium of the following millimolar composition: NaCl, 134; KCl, 5.6; CaCl_2 , 2.2; MgCl_2 , 1.0; NaH_2PO_4 , 1.3; Tris-HCl, 20.0 pH 7.40 at 37°C . The protein concentration was $0.1\text{--}0.2\text{ mg}$ ml^{-1} . After preincubation at 37°C for 30 min with or without 5-hydroxytryptamine (5-HT) at 10^{-6} mol l^{-1} ^3H -5-HT (27.6 Ci mmol^{-1} New England Nuclear) as added to 1.0–3.5 μmol of the membrane suspension. Samples were taken at various time intervals, vacuum filtered through Whatman GF/A filters ($\approx 2.5\text{ cm}^2$) and rinsed with 20 ml of the respective medium. The binding was completed in 10 s and the medium kept cold in order to minimize dissociation of bound ^3H -5-HT. Specific binding was defined as the difference between the binding occurring in the presence of 5-HT at 10^{-6} mol l^{-1} . Radioactivity trapped on filters was measured on Wallac Darcis NTL 214 liquid scintillation counter in aylene based solution (Jensen and McClure 1973) at 34.5% efficiency. Proteins were measured by the method of Lowry *et al.*

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used logarithmically as the abscissa, the linearly presented specific binding fitted a sigmoidal curve (Fig. 1 b). The Scatchard plot (Fig. 1 b, inset) of the 10 s binding values showed a curve and assuming that only one population of binding sites is involved, this is suggestive of positive cooperativity. The corresponding plot of the 60 s binding values, on the other hand, fell on a straight line without any evidence of cooperativity. At $5 \cdot 10^{-6}$ mol l⁻¹ amount bound in 10 s was 38% of that in 60 s, while at $3 \cdot 10^{-4}$ mol l⁻¹ the corresponding percentage was 71. The specific binding taking place in 10 or 60 s both show saturability and both give a maximum of binding sites of about 250–300 fmol mg⁻¹ protein but differ in respect of the shapes of the saturation curves and Scatchard plots. These preliminary results show that the electrolyte composition of the incubation medium, the incubation time and the condition of the membrane preparation markedly affect the binding between 5-HT and its proposed receptor. These factors should be taken into consideration when studying pharmacology and binding characteristics of the receptor.

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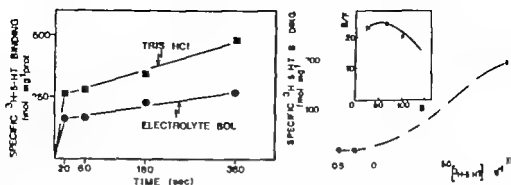


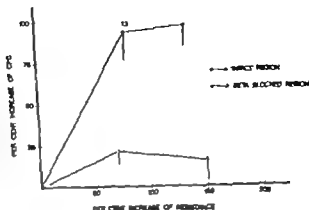
Fig. 1 a. Time course of specific binding of [³H]-5-HT to rat brain synaptic plasma membranes. The concentration of [³H]-5-HT was 10^{-8} mol l⁻¹. ■ 20 mmol l⁻¹ Tris-HCl buffer ● electrolyte solution (see text). Each point is the mean of 5 expts.

Fig. 1 b. Specific binding of [³H]-5-HT to rat brain synaptic plasma membranes. The binding amount 10 s was assayed as described in text. The amount of [³H]-5-HT expressed in fmoles plotted against the concentration of the amine in the medium as the logarithmic function. The inset presents the data as a Scatchard plot, where B denotes the specific binding in fmol mg⁻¹ protein and F is the concentration of [³H]-5-HT in mol l⁻¹. Each point is the mean of 4-6 expts.

(1951). The morphology of the synaptosomal and synaptic plasma membrane fractions as checked Philips 300 EM electron microscope after glutaraldehyde fixation and using conventional techniques specimen preparation. All chemicals used were analytical grade products available commercially.

Imipramine at $3 \cdot 10^{-6}$ mol l⁻¹ had no effect on the binding of [³H]-5-HT to the synaptic plasma membranes. In 3 expts, with imipramine the binding recorded in samples taken between 0 and 10 min of incubation in the electrolyte medium differed less than 5% from controls. Thus, an operative presynaptic uptake mechanism for 5-HT in the preparation used seems unlikely even in the medium containing sodium. The time course of the specific binding of [³H]-5-HT at 10^{-8} mol l⁻¹ and 37°C was different in Tris-HCl buffer 20 or 50 mmol l⁻¹ from that in the electrolyte medium (Fig. 1 a). In both media, however the binding consisted of a fast initial phase completed within 20 s and a slow late phase. The specific binding of [³H]-5-HT in the electrolyte medium was lower and amounted to 59.1 ± 52.1% and 60.4% of the binding in 20 mmol l⁻¹ Tris-HCl buffer after 20, 60, 180 and 360 s respectively (mean values, n = 5). This difference was evident only in fresh preparation but storage for 24 h at 0°C under nitrogen did not affect the specific binding in Tris-HCl buffer but in the electrolyte medium the specific binding was 47% above that observed in a fresh preparation and only 14% below that in Tris-HCl buffer (mean values, n = 3). Thus, the ionic composition of the medium and the storage of the membranes affected the specific binding of [³H]-5-HT. Sodium ions might decrease agonist (5-HT) binding, i.e. 5-HT might have a higher affinity for a nonsodium conformation of the receptor as has been reported for the opiate receptor (Pert and Snyder 1974). Alternatively Tris-HCl might non-specifically alter the conformational structure of the membranes unveiling potential 5-HT binding sites which remain hidden in a physiological electrolyte environment.

The fast initial specific binding of [³H]-5-HT occurring in 10 s did not increase linearly as a function of amine concentration and half maximal binding amounting to some 100 fmol mg⁻¹ protein was achieved at $5 \cdot 10^{-8}$ mol l⁻¹. When the amine concentration was



1 Diagram showing the effect of noradrenaline infusion on CFC (capillary filtration coefficient) in intact muscle before and after blockade. The data are plotted against the concomitant rise of vascular resistance and as mean values \pm S.E. denote number of observations.

muscle upon the pattern of resistance response to noradrenaline was similar but the magnitude of the resistance increase was somewhat more pronounced for a given dose (25%).

The capillary filtration coefficient (CFC), reflecting the number of patent capillaries and, hence, precapillary sphincter tone, was determined in the control state before each period of noradrenaline administration and after 2-3 min of infusion when the evoked resistance response had reached a steady state. Control CFC was quite stable in the individual animal and was unaffected by β -blockade. In the whole material control CFC varied between 0.011 and 0.017 ml/min/100 g mmHg, the mean value being 0.014 ± 0.001 (S.E.) mean/100 g mmHg ($n = 47$). Noradrenaline administration to the intact muscle region caused a dilatation of the precapillary sphincters as evidenced by a clearcut increase in the CFC. The CFC increment above control level ranged between 45 and 160 per cent and showed no consistent relation to the amount of infused noradrenaline. After β -blockade, increase of CFC was much reduced or abolished and in several of the infusion experiments a decrease of the CFC was instead observed. Fig. 1 summarizes the CFC alterations during noradrenaline infusion (classified mean values \pm S.E.), expressed in per cent of the control values prior to infusion and plotted against the concomitant per cent increase of peripheral vascular resistance (increase of resistance by 40-100 and 100-200 per cent, respectively). It can be seen that the mean increase of CFC in the intact muscle region was about 100 per cent at the moderate as well as at the more intense constriction of the resistance vessels. In the β -blocked muscle region there was also an increase of the CFC, on the average, but the increment was much smaller ($p < 0.001$) and amounted only to 19 and 16 per cent, respectively.

Comments

The significant effect of blood-borne noradrenaline on peripheral vascular functions is considered to be vasoconstriction caused by α -adrenoceptor activation. Vasodilatation due to activation of β -adrenoceptors has thus been regarded of little importance since, in the resistance vessels, the β -adrenergic inhibition of vascular tone is greatly overwhelmed by

Noradrenaline evoked beta adrenergic dilatation of precapillary sphincters in skeletal muscle

By

JAN LUNDVALL AND JÄHN HILLMAN

Blood-borne noradrenaline exerts a β -adrenergic dilator effect in the resistance vessels of skeletal muscle, but the inhibition of vascular tone is comparatively weak and is counteracted by the simultaneous much stronger α -adrenergic constriction (e.g. Rapola and Green 1965; Brick, Hutchinson and Roddie 1967; Viveros, Garlick and Renkin 1968). The response evoked by activation of the sympathetic nerve fibres to skeletal muscle includes such a seemingly insignificant β -adrenergic dilator component (Viveros 1968). Recent investigations, however, showed that this neurogenic β -dilator effect is quite marked in the microvessels, especially in the "precapillary sphincters" despite the fact that the total muscle vascular resistance is only little influenced (Lundvall and Järbult 1974). The present study indicates that blood borne noradrenaline evokes a similar marked preferential β -adrenergic dilatation of the precapillary sphincters.

Methods

10 cats anesthetized with chloralose (50 mg/kg b.wt.) and urethane (100 mg/kg b.wt.) were used. The right lower leg muscles were isolated and the preparation was placed in a water-filled plethysmograph (38°C) to permit volumetric determination of the capillary filtration coefficient (Meflender 1960; Lundvall and Järbult 1976). Arterial inflow pressure and venous outflow of blood were continuously recorded. Noradrenaline was administered by loose arterial infusion in doses of 1.0-3.0 μ g/ml. The dose was given before and after regional β -blockade. Each infusion period lasted 6 min with intervals of 8-10 min to permit complete recovery of vascular tone in between the infusions. Propranolol was given close to the end of the infusion (600-800 μ g/kg muscle) to block the β -adrenoceptors and concomitantly venous outflow of blood was discarded to avoid systemic effects. Test experiments using the dextroisomer of propranolol showed that the effect of the β -blocking agent to be reported was not related to an unspecific influence, such as an anesthetic one.

Results

Noradrenaline administration to the muscle vascular bed with intact β -adrenoceptors ("intact region") invariably evoked a constriction of the resistance vessels, which was developed within 90 s and then maintained approximately constant during the infusion period. The steady state increase of regional resistance above control level ranged between 50-200% and was roughly related to the amount of infused noradrenaline. In the β -block-

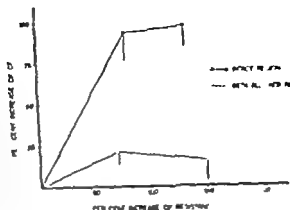


Diagram shows the effect of noradrenaline infusion on CFC (capillary filtration coefficient) in intact muscle before and after blockade. The data are plotted against the concomitant increase of vascular resistance and as mean values \pm S.E. the number of observations.

In the intact muscle region the pattern of resistance response to noradrenaline was similar but the magnitude of the resistance increase was somewhat more pronounced for a given dose (25%).

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Diagram showing the effect of noradrenaline infusion on CFC (per cent of control) in the whole muscle region before and after blockade. The data are cleared against the concomitant rise of vascular resistance and to mean values \pm S.E. of the number of observations.



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Comments

The significant effect of blood-borne noradrenaline on peripheral vascular functions is now considered to be vasoconstriction caused by α -adrenoceptor activation. Vasodilatation due to activation of β -adrenoceptors has thus been regarded of little importance since, in the resistance vessels, the β -adrenergic inhibition of vascular tone is greatly overwhelmed by

the α -adrenergic constriction (*cf* resistance data given above). The present study however strongly indicates that β -adrenergic relaxation is the dominating effect of blood-borne noradrenaline in the precapillary sphincters. In this vascular section, the β -adrenergic inhibitory influence evidently overrides the α -constriction that has been reported to be evoked by noradrenaline (Mellander 1966). Accumulation of "vasodilator metabolites" as a result of the blood flow reduction induced by noradrenaline may contribute to any α -adrenergic constriction of the precapillary sphincters and explain the β -relaxation observed also after β -blockade (Fig. 1).

The observed pronounced β -adrenergic dilatation of the precapillary sphincters in response to noradrenaline infusion was fully developed at doses causing only a moderate increase of vascular resistance (Fig. 1). Preliminary data from an extended series of experiments in fact indicate that the β -adrenergic inhibition of sphincter tone is marked already at such small doses of noradrenaline that hardly affect total muscle vascular resistance. It is further that the noradrenaline induced dilatation of the precapillary sphincters may be as large as that evoked by adrenaline. In the intact organism, catecholamines released by the adrenal glands might exert an important β -adrenergic control of transcapillary exchange as a result of the inhibition of precapillary sphincter tone and the attendant increase in the capillary surface area available for transcapillary exchange. The blood-borne catecholamines may in this respect act in concert with the previously described nervous β -adrenergic control of precapillary sphincter tone (Lundvall and Järhult 1976).

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Kidney volume expansion and prostaglandin release by bradykinin. The effect of indomethacin pretreatment

By

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Abstract

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When bradykinin was infused into the left renal artery in anaesthetized dogs which were loaded with furosemide in saline to produce diuresis of approximately 1 ml/min. Bradykinin initially increased renal blood flow (70%), kidney volume (20%), intracapsular pressure (50%), urine PG-excretion (80%) and decreased urine osmolality (20%). Kidney volume, diuresis and urine osmolality changed equally during continuous saline infusion, while other parameters subsided to control levels. When renal blood flow was maintained constant during the infusion of bradykinin PG-excretion increased (130%), the glomerular filtration decreased (66%) and thus as well as by decreased kidney volume, diuresis/natriuresis. It unchanged urine osmolality. Indomethacin (2.5 mg/kg i.v.) decreased renal blood flow (15%) and inhibited urine PG-excretion, and after indomethacin showed effects which are very similar to those observed before indomethacin treatment. It is concluded that increased intracapsular volume is a sufficient determinant of bradykinin increase of whole kidney volume. The accompanying increase of intracapsular pressure does not appear to contribute to the kinin induced PG-release. The vasodilation by bradykinin seems independent of prostaglandins. The diuresis/natriuresis and decreased urine osmolality are probably of hormonal origin.

Kidney kinins, bradykinin and kallidin (Hjal *et al.* 1976), are potent renal vasodilators reduce natriuretics (Webster and Gilmore, 1964; Heldreich *et al.* 1964; Barracough *et al.* 1965; Wells *et al.* 1969) and increase renal prostaglandin synthesis (McGiff *et al.*

The kinins also increase kidney volume (Sjöström and Berde 1963), which will raise intracapsular pressure due to the low distensibility of the kidney capsule (Hebert *et al.* 1971, Olsen 1977).

In furosemide-diuretic dogs ureteral constriction and kidney compression, both of which have increased the intracapsular pressure to 40 mmHg, have previously been shown to affect the urinary excretion of prostaglandin E-like material (Olsen *et al.* 1976). Since this is a causal relationship between the intracapsular pressure and the rate of urinary PGE excretion one object of the present study was to investigate whether a correlation between

the two parameters existed during renal intraarterial infusion of bradykinin with or without constriction of the renal artery. In addition the possible effects of the prostaglandins released by bradykinin was studied by using indomethacin as a tool to inhibit renal prostaglandin synthesis.

Materials and methods

12 female mongrel dogs (12-25 kg) which had been fasted overnight, were used for the experiments. They were kept on a standard diet (Doggy®) with free access to water.

The dogs were anesthetized with pentobarbital sodium (30 mg/kg i.v.) and intubated for spontaneous respiration. A Statham (P23Db) transducer was connected to a femoral artery to measure blood pressure and a catheter was placed in the femoral vein to permit subsequent infusion. The abdomen was opened by a midline incision. The left ureter was cannulated for urine collections and a flow probe was placed around the left renal artery for the measurement of renal blood flow (electromagnetic flow meter, Veeco®).

A 25-gauge needle attached to a polyethylene catheter was introduced into the left renal artery to the probe and used for intrarenal infusions. Patency was ensured by a continuous infusion of saline at a rate of 0.2 ml/min. In 6 of the experiments a thread suture for manual constriction was also placed around the left renal artery to maintain renal blood flow constant during the kidney infusion. The left kidney was prepared for the measurements of kidney volume (mercury strain gage, connected to a model 28 piezomograph, Parks Elec. Lab.) and subcapsular pressure (P 19 miniature transducer, K&B, Copenhagen). Blood pressure, renal blood flow, kidney volume and subcapsular pressure were continuously recorded on a Gram 7C polygraph. At the end of the experiment the dog was killed by a lethal dose of pentobarbital. Subcapsular pressure recorded at this stage was used as zero value. The kidney was isolated, arterial vessels were ligated. The piezomograph was then calibrated by stepwise arterial infusions of saline.

When the continuously measured parameters had stabilized 15-30 min after surgery one group of dogs was loaded initially with 100 ml 10% mannitol in saline (10 ml/min) and 2 ml/min was infused throughout the experiment. When urine flow had stabilized 100 ng/kg/min bradykinin (Sandoz) was infused into the renal artery for 30 min, and urine was collected for periods of 15 min before, during and after infusion.

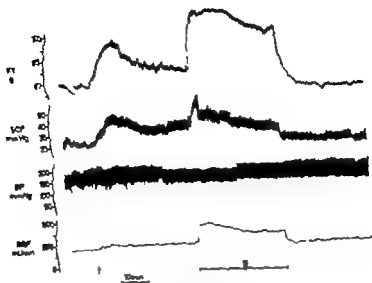
In a second group of 6 dogs the loading procedure was initiated immediately following completion of surgery. When urine flow had stabilized 100 ng/kg/min bradykinin was infused into the renal artery 12-15 min with and without renal artery constriction in random sequence. These experiments were controlled by indomethacin (Dumex) administration (2.5 mg/kg i.v.) followed by 100 ng/kg/min bradykinin for 30 min later. Urine was collected for 10 min before and during the infusions.

Analyses

Urine sodium was determined by flame photometry and creatinine by Jaffé reaction. Urine osmolality was measured with a semimicro osmometer (Kauver model M). Urinary excretion of prostaglandin E₂ material was measured by bioassay. Urine was acidified (pH 3) with 5% formic acid and extracted with chloroform. The chloroform phase was partially evaporated and the residue chromatographed on silica acid (0.5 g Biosil®). Non polar lipids were eluted with chloroform while prostaglandins are eluted with chloroform-methanol (1:8). The PGE like activity was assayed on a rat fundus strip which is especially sensitive to prostaglandin E₂ (Splawinski *et al.* 1973, Crutchley and Piper 1975). The specificity of the assay procedure was further assured by the use of Krebs-Henseleit solution with a mixture of substances containing (in µg/ml) methysergide 0.2, propranolol 3.0, phenoxymethazine 0.1, atropine 0.1 and phenhydramine 1. Recoveries of added prostaglandin E₂ (Unilever) were $85 \pm 2\%$ (N=10). After acid chromatography selected samples were spotted on precoated silica plates for thin layer chromatography. The plates were developed in the solvent system chloroform-methanol:acetic acid (100:10:1, 0.65). After reanal division the plates were scraped off into plastic tubes and it was assured that the majority (>90%) of the biologically (rat fundus strip) active material co-chromatographed with the PG standard.

Statistics

The results are presented as mean \pm S.E. The significance of the changes was assessed by Student's *t* on paired comparison after a logarithmic transformation.



The loading procedure was started at A, and 100 $\mu\text{g/kg/min}$ bradykinin was infused into the renal artery at B. KV = kidney volume, SCP = subcapsular pressure, BP = femoral artery blood pressure and renal blood flow.

Results

1 shows the effect of the loading procedure on the continuously registered parameters representative expt. When diuresis was stable, (Table I) there were significant increases kidney volume (7%), subcapsular pressure (31%) and systemic mean blood pressure (%). Renal blood flow did not change significantly. Fig. 1 shows the effect of 100 $\mu\text{g/kg}$ bradykinin subsequently infused into the renal artery. Renal blood flow, kidney volume, subcapsular pressure immediately rose to maximum levels 1-2 min after the infusion started. Renal blood flow and subcapsular pressure then fell towards control levels but there was no significant difference between the kidney volume expansion in the early and the late phase of the infusion compared with the final phase (Table II). Systemic blood pressure (Table I) remained unchanged during the infusion.

Bradykinin significantly increased urine volume, sodium and PG-excretions, and reduced osmolarity (Table II and III). The increase in sodium and PG-excretions was greater the first 15 min period of a sustained kidney infusion than in the second (Table II).

TABLE I. The effect of extracellular volume expansion on blood pressure and kidney parameters. Results are mean \pm S.E. (N = 6)

	Before expansion	During sustained expansion	P
kidney volume (g)	76 \pm 5	81 \pm 8	0.05
subcapsular pressure (mmHg)	38 \pm 3	51 \pm 3	<0.05
renal blood flow (ml/min)	179 \pm 30	177 \pm 28	n.s.
renal pressure (mmHg)	123 \pm 6	141 \pm 7	<0.02

TABLE II The effect of bradykinin on kidney parameters during enhancement of renal blood flow. 100 ng/kg/min bradykinin was infused into the renal artery from 0 to 30 min. Results are mean \pm S.E. (N=6). Symbols in column represent comparisons between the two infusion periods (I-II or I-III or I-IV or II-III or II-IV or III-IV). Otherwise the results have been compared with preinfusion levels (-15 to -5 min). n.s. = not significant $-p < 0.05$ $-p < 0.01$ $-p < 0.001$

min	+15-0	0-15	15-30		
Kidney volume (ml) g	81 \pm 8	97 \pm 8	96 \pm 8	n.s.	81 \pm 9
Subcapsular pressure mmHg	21 \pm 3	39 \pm 6	31 \pm 4		19 \pm 2
Renal blood flow ml/min	177 \pm 28	299 \pm 53	258 \pm 37		173
Diuresis ml/min	1.0 \pm 0.1	1.8 \pm 0.2	1.8 \pm 0.1	n.s.	1.2 \pm 0.1
Sodium excretion μ eqv/min	31 \pm 5	103 \pm 29	70 \pm 18		25 \pm 6
Urine osmolality mosm/l	622 \pm 39	487 \pm 23	444 \pm 39	n.s.	625 \pm 3
PG-excretion ng/min	2.4 \pm 0.8	12.3 \pm 4.9	7.6 \pm 2.5		21 \pm 1

No significant correlations were observed between kinin induced changes of renal blood flow—subcapsular pressure ($r = 0.24$), subcapsular pressure—urine PG excretion ($r = 0.1$), renal blood flow—urine osmolality ($r = 0.29$), urine PG excretion—urine osmolality ($r = 0.1$) or renal blood flow—sodium excretion ($r = 0.24$). The levels of each of these parameters after kinin infusion were significantly correlated with the control levels (Table IV).

TABLE III The effect of bradykinin on kidney parameters during constant renal blood flow and indomethacin treatment. 100 ng/kg/min bradykinin was infused I to the renal artery and renal blood flow is allowed to increase (II) or is maintained constant (III). At (V) bradykinin is infused after the administration of 2.5 mg/kg indomethacin I. Results are mean \pm S.E. (N=6). n.s. = not significant, $-p < 0.05$ $-p < 0.01$ $-p < 0.001$

	Non-pretreatment			Statist. assess.	Indomethacin pretreatment				
	Control Kinf infusion				Control	Statist. assess.	Kinf infusion		
	I	RBF increase II	RBF constant III	III/I	III/II	IV	IV/I	V	Statist. assess.
Subcapsular pressure mmHg	20±3	36±4	10±2			11±2		31±6	n.s.
Renal blood flow ml/min	155±22	64±36	170±31	n.s.		101±17		192±28	n.s.
Diuresis ml/ml	1.6±0.3	2.9±0.4	0.9±0.3			1.3±0.2	n.s.	2.6±0.3	n.s.
Sodium excretion μeqv/min	81±6	245±36	35±24			45±17	n.s.	163±39	n.s.
Creatinine excretion μmol/min	1.7±0.4	2.0±0.5	0.7±0.2						
Urine osmolality mosm/l	607±73	440±35	533±89	n.s.		550±71	n.s.	428±52	n.s.
PG-excretion ng/min	1.6±0.7	7.9±4.3	6.9±2.1		n.s.	<		<	

V. Correlations between bradykinin induced changes of some kidney parameters and their control levels. Kain induced changes of renal blood flow and subcapsular pressure represent area levels observed during the first 10-15 min of infusion. (N=12.)

var	Correlation coefficient	Regression line	P
blood flow	0.95	$y = 1.70x - 0.01$	<0.001
subcap pressure	0.64	$y = 0.99x + 17.11$	<0.05
urine	0.76	$y = 1.18x + 0.89$	<0.02
urine excretion	0.70	$y = 156x - 82.57$	0.05
osmolality	0.72	$y = 0.41 + 192.31$	<0.05
creatinine	0.82	$y = 4.57x + 1.51$	0.01

p. 2 and Table III show the effect of 100 ng/kg/min bradykinin infused into the renal artery during constant renal blood flow. The urine creatinine excretion decreased to approximately 40% of the control level and significant reductions of diuresis, sodium excretion and subcapsular pressure were observed. Further Table III shows that the arterial traction significantly changed the effects of the kalin infusion on all the parameters except the urine prostaglandin excretion.

domethacin treatment (Table III, IV Fig. 2) significantly reduced renal blood flow to approximately 65% of control. This was paralleled by decreased subcapsular pressure, as prostaglandin excretion decreased below the range of sensitivity of the assay procedure (ng/min). The changes of diuresis, sodium excretion or urine osmolality were neither distinct nor significant. 100 ng/kg/min bradykinin produced a diuretic/natriuretic response after indomethacin treatment which was not significantly different from that observed before indomethacin treatment. The level of renal blood flow during kalin infusion after indomethacin treatment was, on the other hand, significantly lower than that observed before indomethacin treatment, but there was no significant difference between the increases in renal blood flow produced by the kalin infusion before or after indomethacin administration.



Fig. 2. 100 ng/kg/min bradykinin was infused into the renal artery at A, B and D. Renal blood flow was measured constantly during A, and 2.5 mg/kg indomethacin was administered at C. BP = femoral artery blood pressure, SCP = subcapsular pressure and RBF = renal blood flow.

Discussion

In the present expts. the renal intraarterial bradykinin infusion produced a marked sustained expansion of the kidney volume. This was accompanied by an increased capsular pressure (expansion pressure) which, however declined toward the control level during sustained enhancement of kidney volume, presumably as a result of changes in capsular distensibility (Hebert and Arbus 1971 Olsen 1977). The most important effect of kidney volume expansion will probably derive from the accompanying enhancement of subcapsular pressure which reflects an increase of the interstitial pressure (Hebert and Arbus 1971).

In similar experimental conditions urinary prostaglandin excretion has been found to increase during enhancement of the intrarenal pressure (Olsen *et al* 1976). Yet, the increase in urine prostaglandin excretion by bradykinin in expts. where the subcapsular pressure increased (renal blood flow increased) and those where the subcapsular pressure decreased (renal blood flow maintained constant) indicates that changes of intrarenal pressure apparently is not an important factor in the kinin induced prostaglandin release. The results thus accord with the concept that bradykinin releases prostaglandins by a direct chemical activation of an acyl hydrolase (phospholipase) (Kunze and Vogt 1971).

Renal i.a. infusion of bradykinin has previously been shown dose-related (5-500 µg/min) to increase renal blood flow and urine electrolyte excretion without affecting the glomerular filtration rate in pentobarbital anesthetized and non-loaded or saline loaded dogs (Webster and Gilmore 1964 Herdenreich *et al* 1964 Barraclough and Mills 1965, 1966 *et al* 1969). During constant renal blood flow bradykinin decreased urine creatinine excretion which accords with the decreased inulin clearance (glomerular filtration rate) observed by Willis *et al* (1969).

The ability of bradykinin to increase sodium excretion and to decrease urine osmolality is presumably related to the enhancement of renal blood flow since these effects disappeared when renal blood flow was maintained constant. Noticeable was the observation that the effect of bradykinin on kidney parameters (Table IV) including the urine prostaglandin excretion depended so much upon the control levels.

Exogenous prostaglandin E is usually a potent vasodepressor and natriuretic substance. In the present expts. the level of renal blood flow during bradykinin infusion was kept lower after indomethacin pretreatment than before such treatment. This could suggest that prostaglandins released by bradykinin amplified the vasoactivity of the peptide. This interpretation was advanced by McGiff *et al* (1975) In isolated canine kidney these authors observed a decreased level of renal blood flow evoked by bradykinin, when the kidney was pretreated with indomethacin. Indomethacin alone, however markedly reduced renal blood flow (Table III., McGiff *et al* 1975) The lower level of renal blood flow during bradykinin infusion after indomethacin pretreatment might thus alternatively be explained by a lowered preinfusion level of renal blood flow induced by indomethacin, since a significant relationship was found to exist between preinfusion and kinin infusion level of renal blood flow in non-pretreated expts. (Table IV).

One might speculate why indomethacin pretreatment in the present expts. failed to

encing effects of the prostaglandins released by bradykinin. Bradykinin and prostaglandins however share very similar effects on renal hemodynamic and excretory functions. The noted effect of bradykinin at the infusion dose used in the present exper. might perhaps conceal possible effects of intrarenally released prostaglandins. It might therefore be more appropriate to investigate the renal kinin-prostaglandin interrelationship in conditions where intrarenally increased kinin activity mediates the prostaglandin release. Turner and Berde (1963) observed that kidney volume expansion accompanied the enhancement of renal blood flow evoked by bolus kinin injections. In the present results 2 observations suggest that the kinin induced enhancement of renal blood flow is not the only determinant of kidney volume changes. Thus kidney volume remained equally expanded during sustained infusion of bradykinin, although the enhancement of renal blood flow significantly decreased, and subcapsular pressure (kidney volume) decreased during the infusion when renal blood flow was maintained constant. Kidney volume and urine flow however changed in parallel, which might imply that tubular volume is an important factor to determine whole kidney volume (Ouvrik *et al.* 1971, Olsen 1977). The results are complementary to those of Chibard *et al.* (1965). Using *in situ* dog kidneys these authors demonstrated that changes of renal blood flow were closer related to changes of the creatinine distribution space than to changes of the vascular volume. Bradykinin has been shown to increase capillary filtration and interstitial fluid (lymph) production in several vascular beds (Haddy *et al.* 1970). The significance of this in relation to kidney volume expansion is negligible, since kidney volume decreased during the kinin infusion when renal blood flow was maintained constant.

Two other observations in the present study confirm that kidney volume may be related to both changes of renal blood flow and/or diuresis. Thus, kidney volume and diuresis decreased during the loading procedure although renal blood flow did not change significantly while kidney volume and renal blood flow decreased after indomethacin treatment without significant changes of urine flow.

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A quantitative study of the insulin release induced by vagal stimulation in anesthetized cats

By

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Abstract

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Insulin release by vagal stimulation in anesthetized and cannulated cats. The plasma insulin concentration and blood flow in the portal vein were determined continuously and the insulin output was calculated. Stimulation of either the right or the left cervical vagus released the same amount of insulin, rate bilateral stimulation released twice as much. Following stimulation that depleted the "vagal-insulin pool" a recovery period of 15-20 min was needed before the same maximal output could be used again. With shorter interstimulatory periods the amounts of insulin released were reduced. When more than 2-3 000 impulses were applied during a stimulation period, the amount of insulin released per unit was constant. Atropine (0.3-2 mg/kg) did not reduce the vagally-induced insulin release.

Studies in which the vagal nerves have been stimulated electrically indicate that such stimulation releases insulin (Kaneio *et al.*, Frohman *et al.* 1967; Daniel and Henderson 1967). In acute experiments on cats quantitative estimations of vagally-induced insulin output were easily made by concomitantly recording the blood flow and the plasma insulin concentration in the portal vein (Uynäs-Wallén and Nilsson, *in press*). The release mechanism was found to be easily fatigued on stimulation with frequencies above 3-4 Hz. Irrespective of frequency or duration of stimulation, the release declined as soon as a total of 2-3 000 pulses had been applied. The existence of an insulin pool, available for immediate release, and amounting to approximately 2% of the total pancreatic insulin store, was suggested. The present study was undertaken to obtain further quantitative information on vagally-induced insulin release. These studies concern the effect of unilateral and bilateral vagal stimulation, the characteristics of the release mechanism as to duration, fatigue and recovery. In addition the relation between number of impulses applied and insulin release response were investigated as well as the effect on insulin release by atropine.

Methods

Experiments were performed on cats that had been fasted for 18 h. The cats were anesthetized with chloralose (50 mg/kg) and urethane (100 mg/kg). The incision below the diaphragm and the spleen was re-

TABLE I Amounts of insulin released following stimulations of either the right or the left vagal nerve in cats. R = right, L = left.

Exp. No	Number of impulses	Insulin released μ	
1	1 500	410	R
	1 500	470	L
2	2 400	150	R
	2 400	190	L
3	2 400	1 540	R
	2 400	1 320	L
4	3 600	4 850	R
	7 200	4 890	R
	9 000	4 800	L

moved. The portal vein was ligated close to its bifurcation and the distal stump of the superior vein was cannulated. The blood draining from the stomach, the pancreas and the duodenum vein of the cat, passed through a drop-chamber unit and returned to the cat via a cannulated femoral vein.

The cervical vagal nerves were stimulated uni- or bilaterally with a Grass stimulator (SS1) giving pulses of 7-10 V 2-3 ms duration and 1-20 Hz. A more detailed description of the surgical procedure been presented elsewhere (Uvnäs-Wallensten and Nilsson, *in press*). Plasma insulin concentration measured by radio-immunoassay as described by Nilsson and Uvnäs-Wallensten (1974). The insulin output was calculated by multiplying portal insulin concentration and plasma flow. The vagally-stimulated insulin release was determined by subtracting the basal insulin release from the total insulin output during stimulation of the nerves.

Results

Unilateral and bilateral stimulation

Stimulation of either the right or left vagal nerve ($n=4$) released similar amounts of insulin provided that the same number of impulses were delivered (Table I).

When bilateral and unilateral stimulations of the same frequency and duration were performed in a cat ($n=4$), bilateral stimulation released about twice as much insulin into portal blood as did unilateral stimulation (Fig. 1).

As shown in Fig. 2, a maximal insulin response could be induced by stimulation of

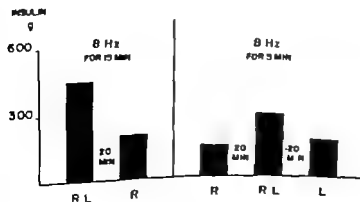
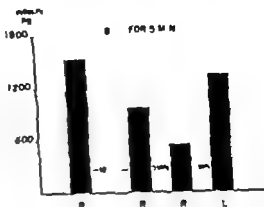


Fig. 1 2 expts. demonstrating release of insulin following lateral and bilateral stimulation (8 Hz for 5 and 15 min). Note the additive effect of bilateral stimulation.



Expt. demonstrating the fatigue of insulin release following repeated supramaximal stimulations of either the right or the left vagus (5 Hz for 5 min, 2 400 impulses) with 15 min interstimulatory periods. Note that stimulation of the left nerve could not elicit amounts of insulin immediately following stimulation of the right nerve.

vagal nerve immediately following supramaximal stimulation of the right vagal nerve, when the release mechanism of the right nerve was fatigued ($n=3$).

Recovery of release mechanism

After a supramaximal vagal stimulation ($>2\ 300$ impulses) a subsequent supramaximal stimulation released smaller amounts of insulin, unless the release mechanism was allowed a recovery period of 15–20 min ($n=4$). The shorter the interstimulatory period, the smaller amount of insulin released by subsequent stimulations. The fatigue phenomenon is treated in Fig. 2.

Insulin release per impulse

Due to the rapid fatigue of the insulin release mechanism, the output of insulin per impulse is difficult to estimate. However, on submaximal stimulation (less than 3 000 impulses), a constant relationship was found between the amount of insulin released and the number of impulses applied, regardless of the frequency or duration of stimulation (Fig. 3 a, b, = Table II).

Effect of atropine on vagally-stimulated insulin release

Identical unilateral vagal stimulations (5 Hz for 10 min) were performed in 11 different experiments. The interstimulatory period was 30 min. In 6 experiments, atropine (0.2–2 mg/kg) was administered 10 min before the second stimulation. The insulin release following the second stimulation was expressed as a percentage of that caused by the first stimulation. In the other 5 experiments, the insulin release following the second stimulation was 102% of that after the first (86–124). Atropine did not reduce the insulin output within the dosage range tested. On an average, the insulin release caused by the second stimulation was 105% of that induced by the first (Table III).

Discussion

In a previous paper (Uvnäs-Wallensten and Nilsson 1975) the existence of an insulin pool in the pancreas, depletable by vagal stimulation, was suggested. The background to this pro-

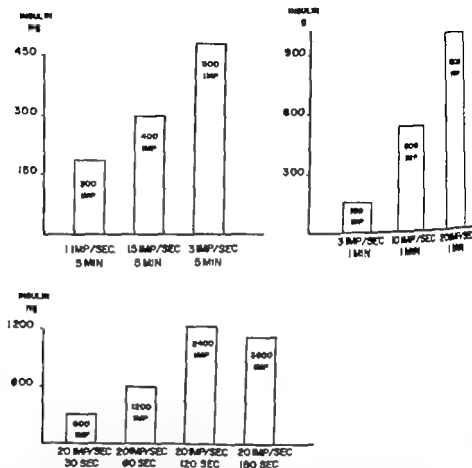


Fig. 3 a, b, c. 3 expts. demonstrating that the amount of insulin released per impulse is fairly constant following vagal stimulation with less than 2 3 000 impulses. Either the frequency (a and b) or duration of the stimulation (c) was varied. The insulin/impulse ratio declined when more than 3 000 impulses were applied (c) reflecting the fatigue of the release mechanism. Interval between stimulations 25 min (Table II).

posals was the observation that the insulin release mechanism proved to be easily fatigued following vagal stimulation. Due to the long recovery period required (15–20 min), it was considered unlikely that a cholinergic neurotransmission failure was responsible for the fatigue phenomenon.

The results of the present study suggest that unilateral supramaximal stimulation of either the right and the left vagi causes depletion of such an insulin pool, and that stimulation of either of the nerves releases the same amount of insulin. Bilateral stimulation yields more insulin than does unilateral stimulations, the output being approximately doubled. If unilateral release mechanisms seem to operate independently of each other since the output in response to a supramaximal stimulation of one vagal nerve is maximal when the contralateral release mechanism has not yet recovered from a previous stimulation.

The explanation of the additive effect of bilateral vagal stimulation and the independent behaviour of the vagal release mechanism is not clear. The two vagal nerves might innervate separate beta-cell populations. If this is the case, more insulin will be released on bilateral

II Insulin release on vagal stimulation with different frequencies or durations showing the output of insulin/impulse. Intervals between stimulations—20 min (see Fig. 3).

Frequency Hz	Duration	Number of impulses	Insulin release μg	μg/ imp.
1	300	300	200	0.7
1.5	300	450	310	0.7
3	300	900	520	0.6
3	60	180	160	0.9
18	60	600	530	0.9
20	60	1 200	1 000	0.8
20	30	600	310	0.5
28	60	1 200	600	0.5
28	120	2 400	1 40	0.5
28	180	3 600	1 300	0.3

dation, because more beta-cells are reached by the vagal impulses. Alternatively each cell might have a dual innervation, unilateral stimulation releasing only part of the in pool, accessible for vagal release. A sustained release of insulin, corresponding to rate of refilling of the "pool" should be expected to occur when vagal stimulation is used after depletion of the pool. Such a continuous insulin secretion has not been observed. Therefore, it cannot be excluded that electrical vagal stimulation not only promotes the release of insulin from the beta-cells, but also activates a mechanism inhibiting in release.

the same cat the insulin release per impulse following repeated submaximal stimulation 3 000 impulses) was found to be constant. This fact makes the cat preparation well suited for testing substances which may influence vagally-stimulated insulin release. Atropine, even in very high doses, did not inhibit the vagally-induced release of insulin. This may indicate that the vagal stimulation was not mediated by acetylcholine or that the acetylcholine receptors are not muscarinic.

III. Two successive vagal stimulations were performed at 5 Hz for 10 min in 11 cats. In cats number 1-4, atropine in doses ranging from 0.2-2 mg/kg was given 10 min before the second stimulation. The insulin output induced by the second stimulation is expressed as percentage of the preceding control stimulation. The interstimulatory period was 30 min.

No.	Atropine mg/kg	Insulin release induced by the second stimulation expressed as percentage of that induced by the first (control) stimulation
	0.2	130
	0.2	120
	0.5	70
	0.5	80
	1	110
	2	120
	—	105 (70-130)
	—	102 (86-124)

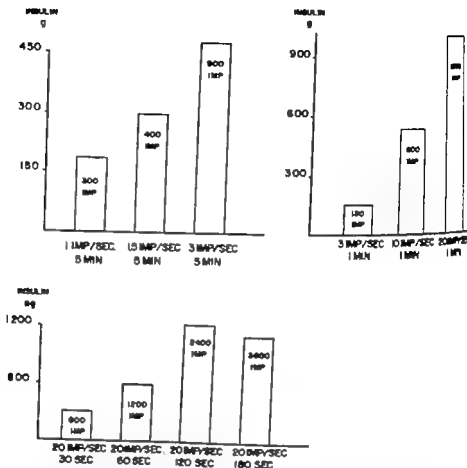


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Indirect vascular actions of (Gln⁸)-neurotensin in canine adipose tissue

By

SUNE ROSELL, ÅKE RÖKAUS, DING CHANG and KARL FOLKERS

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Abstract

EL, S., Å. RÖKAUS, D. CHANG and K. FOLKERS. *Indirect vascular actions of (Gln⁸)-neurotensin in canine adipose tissue* Acta physiol. scand. 1978. 102. 143-147

The vasoconstrictor action of the tridecapeptide (Gln⁸)-neurotensin has been studied in subcutaneous adipose tissue in the regional vessels of anesthetized dogs. Close intra-arterial infusion of (Gln⁸)-neurotensin, 30-120 pmol kg⁻¹ b.wt. min⁻¹ elicited similar vasoconstrictions in the adipose tissue on the infused side and on the contralateral side. This suggests that (Gln⁸)-neurotensin must enter the general circulation before it can elicit vasoconstriction. Removal of parts of the gastrointestinal tract did not alter the vasoconstrictor response. Thus, there is no indication of release of vasoactive substances from the gastrointestinal tract by (Gln⁸)-neurotensin. Infusion into the portal vein elicited the same vasoconstriction in adipose tissue as the same dose administered i.v. It is suggested that the vasoconstrictor action in adipose tissue is not caused by (Gln⁸)-neurotensin *per se*. Instead, vasoactive substance(s) is released from (Gln⁸)-neurotensin.

Neurotensin is a tridecapeptide which has been isolated from bovine hypothalamus (Carraway and Leeman 1973). The amino acid sequence has been established and the synthesis described (Carraway and Leeman 1975 a, 1975 b). Recently Folkers *et al.* (1976) suggested that the acid analog (Gln⁸)-neurotensin rather than neurotensin may be the naturally occurring peptide. In dogs, neurotensin and (Gln⁸)-neurotensin infused i.v. (pmol kg⁻¹ min⁻¹ or more) have pronounced effects on certain functions in the gastrointestinal tract. Thus, they inhibit the gastric motor activity (Andersson *et al.* 1977) and the gastric acid secretion (Andersson *et al.* 1976).

The neurotensins also have vasoconstrictor action in some vascular beds, including innervated adipose tissue (Rosell *et al.* 1976). The vasoconstriction is delayed in onset, starting about 5 min after the beginning of i.v. infusion. Therefore, it was suggested that this effect and possibly other actions of neurotensins, may be mediated by a neurotensin metabolite or by the release of some vasoactive substance(s) (Rosell *et al.* 1976). In the next investigation these possibilities as far as the vasoconstriction in adipose tissue is concerned have been analyzed experimentally.

Frohman *et al* (1967) reported that the atropine blocks the insulin secretion by vagal stimulation in the dog. It is difficult to evaluate their results since inhibition and since they did not determine the effluent blood flow from the pancreas which have fluctuated.

The ability of atropine (0.2 mg/kg) to inhibit the insulin release caused by sham feeding in dogs (Nilsson and Uvnäs-Wallén 1974) may appear inconsistent with our results. However when high doses of quaternary anticholinergic drugs, which do not cross the blood-brain barrier were given instead, the insulin release in response to sham feeding was not affected at all (Uvnäs-Wallén and Andersson, to be published). This probably blocks central neuronal pathways, which are involved in the induction of sham feeding response.

The quantitative role of vagal stimulation in insulin release is not fully understood in anesthetized dogs. Frohman, Erdinl and Javid (1967) recorded a drop in the basal level in the portal blood following surgical vagotomy. However other observations indicate that vagal excitation is of little or no importance for the basal release of insulin. Thus vagotomy does not lower basal plasma insulin levels in the rat (Håkansson, Liedberg and Lundquist 1972) or monkey (Miller 1970). No decline in the insulin output was observed in our own studies on cats following vagotomy.

Vagal activation *per se* released insulin, as shown by the increased insulin levels following sham feeding and following electrical stimulation of the vagal nerves. Moreover, vagal impulses have been shown to influence the insulin output induced by releasing agents. For example, the insulin release in response to glucose administration is reduced in vagotomized rats (Håkansson, Liedberg and Lundquist 1971). In addition, glucose-induced release of insulin *in vitro* is enhanced by cholinergic substances (Culbert and Cook 1974).

Thus, the activation of the vagal nerves, that occurs in connection with meals, causes direct release of insulin, but may also potentiate or modify the release of insulin by humoral factors, such as gastrointestinal hormones or digestive products (e.g. amino acids).

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Fig. 2. Experimental record illustrating blood pressure and blood flow in a dog. (Glu¹²)-neurotensin, 30 pmol kg⁻¹ min⁻¹ was infused intravenously (upper record) the portal vein (lower record).

dorsal viscera. The reference pattern was unaffected by splenectomy (2 expts.), necrectomy (3 expts.), removal of duodenum, jejunum, ileum or colon individually or in those expts. where the whole small intestine was removed the responses were diminished although the latency and vasoconstrictor pattern was unchanged.

Infusion of (Glu¹²)-neurotensin into the portal vein. (Glu¹²)-neurotensin infused into the portal vein in doses ranging between 30–120 pmol kg⁻¹ min⁻¹ produced vasoconstrictor response in adipose tissue which was of the same magnitude as the vasoconstriction following intravenous administration. Moreover the latency and the duration of the vasoconstriction were similar irrespective of the routes of administration (Fig. 2). The vasoconstriction, expressed as the area under the peripheral resistance time curve, was not significantly different from that following the same dose administered intravenously (Student's *t*-test for paired observations, *P* > 0.05) (Fig. 3).

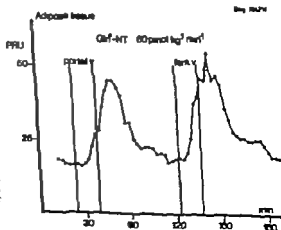


Fig. 3. Changes in vascular resistance in adipose tissue in one dog showing infusion of (Glu¹²)-neurotensin, 60 pmol kg⁻¹ min⁻¹ intravenously or

Methods

The experiments were performed on 19 female mongrel dogs weighing 9–25 kg, anesthetized with pentobarbital, 30 mg/kg with supplement as necessary. Tracheotomy was performed, and the dogs were ventilated with a Braun Melsungen model 74052 respirator. To prevent coagulation, heparin (250 IU) was administered. Subcutaneous adipose tissue in the left and the right inguinal regions was isolated from skin and other surrounding tissues (Rosell 1966). This procedure provides two almost identical adipose tissue preparations, each supplied by one artery, one vein and one mixed nerve. The tissues were not denervated. Blood flow was measured using a silicon-filled drop recorder inserted into the arterial outflow of the tissue. Systemic blood pressure was measured in a carotid artery with a Statham P23Ac transducer and recorded together with blood flow on a Grass model 7B polygraph. Vasoconstriction is given in peripheral resistance units (PRU $\text{mmHg} \times \text{ml}^{-1} \text{min}^{-1} \times 100 \text{ g}^{-1}$). To obtain a measure of the net vasoconstrictor effect of infused drugs in adipose tissue the area under the peripheral resistance curve was calculated using the trapezoidal rule.

Drugs were administered by close intra-arterial infusions via a side arm in the arterial line just proximal to the drop counter. Infusions into the portal vein were made via a polyethylene cannula inserted through mesenteric vein. (Gln¹)-neurotensin (M W 1674) was dissolved in saline.

Results

Adipose tissue blood flow Infusion of (Gln¹)-neurotensin intrarterially (30–120 pmol $\text{kg}^{-1} \times \text{min}^{-1}$) to the inguinal subcutaneous adipose tissue induced vasoconstriction after latency of about 5 min. As is evident from Fig. 1. L.a. infusion of (Gln¹)-neurotensin on one side induced similar vasoconstrictions on the infusion side and contralateral side as far as latency, degree and duration of vasoconstriction are concerned. These results indicate that (Gln¹)-neurotensin must enter the general circulation before inducing vasoconstriction in adipose tissue.

Partial removal of the gastrointestinal tract One possible mechanism for the vasoconstrictor action of (Gln¹)-neurotensin is that the polypeptide releases vasoactive substance. In order to examine this possibility abdominal organs were extirpated in acute experiments and the effects of these procedures on the vasoconstriction in subcutaneous adipose tissue were investigated. (Gln¹)-neurotensin was infused i.v. before and after the extirpations of various



Fig. 1 Experimental record illustrating blood pressure and blood flow in actively denervated subcutaneous adipose tissue. Blood flow was measured in both the left (upper record) and the right (lower record) adipose tissue preparations. (Gln¹)-neurotensin, 120 pmol $\text{kg}^{-1} \text{min}^{-1}$ was infused into the artery of the right adipose tissue.

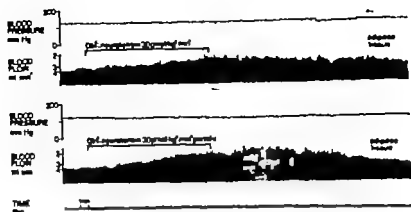


Fig. 2. Experimental record illustrating blood pressure and blood flow in acutely denervated subcutaneous adipose tissue. $(\text{Gln}^1)\text{-neurotoxin}$, $30 \text{ pmol kg}^{-1} \text{ min}^{-1}$ as infused intravenously (upper record) the portal vein (lower record).

abdominal viscera. The reference pattern was unaffected by splenectomy (2 expts.), adrenalectomy (3 expts.), removal of duodenum, jejunum, ileum or colon individually. However in those expts. where the whole small intestine was removed the responses were abolished although the latency and vasoconstrictor pattern was unchanged.

Infusion of $(\text{Gln}^1)\text{-neurotoxin}$ into the portal vein. $(\text{Gln}^1)\text{-neurotoxin}$ infused into the portal vein in doses ranging between $30\text{--}120 \text{ pmol kg}^{-1} \text{ min}^{-1}$ produced vasoconstriction in adipose tissue which was of the same magnitude as the vasoconstriction following intravenous administration. Moreover the latency and the duration of the vasoconstriction were similar irrespective of the routes of administration (Fig. 2). The vasoconstriction, expressed as the area under the peripheral resistance time curve, was not significantly different from that following the same dose administered intravenously (Student's *t*-test for paired observations, 6) (Fig. 3).

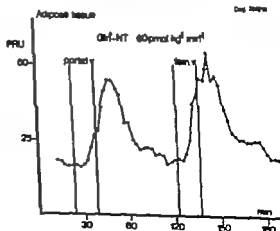


Fig. 3. Changes in vascular resistance in subcutaneous adipose tissue in one dog following infusion of $(\text{Gln}^1)\text{-neurotoxin}$, $60 \text{ pmol kg}^{-1} \text{ min}^{-1}$ intravenously or into the portal vein.

Discussion

Previously reported experiments have shown that in doses above $18 \text{ pmol} \cdot \text{kg}^{-1}$ i.v. both neurotensin and (Gln)-neurotensin elicit a delayed vasoconstriction in domestic canine subcutaneous adipose tissue and to a lesser degree in the skin and small intestine. Due to the long latency of about 5 min following i.v. administration, it was suggested that the vasoconstriction in adipose tissue was indirectly mediated. The vasoconstriction was blocked by adrenergic α -receptor blocking agents or by bilateral ligation of the renal pedicle. Thus, the vasoconstriction does not seem to be mediated by catecholamines or by effect on the renin-angiotensin system or other renal mechanisms. Furthermore, the vasoconstrictor action does not seem to be mediated via the central nervous system since infusion of neurotensins into the vertebral artery produced smaller effects than similar doses administered i.v. (Rosell *et al* 1976).

The present experiments were aimed at further testing the hypothesis of an indirect action of (Gln)-neurotensin. Apart from a slight initial vasodilatation, close intra-arterial infusion of (Gln)-neurotensin to the inguinal adipose tissue did not induce any changes in peripheral resistance that could be ascribed to a direct action of the peptide. Instead i.a. infusions of (Gln)-neurotensin on one side produced similar vasoconstrictions on the infusion side and contralateral side. This result indicates that (Gln)-neurotensin must enter the general circulation before it can elicit vasoconstriction in the adipose tissue. Therefore it is possible that (Gln)-neurotensin releases some vasoconstrictor substance or alternatively is metabolized to one or more peptides with vasoconstrictor properties. Both these alternatives have been considered in the present series of experiments. The results of experiments in which abdominal organs or parts of the gastrointestinal canal were extirpated did not indicate any release. The reduction of the vasoconstrictor response following extirpation of large parts of the gastrointestinal tract may be a consequence of the decrease in the portal blood flow and thus an altered distribution of the infused (Gln)-neurotensin.

The finding that infusion of (Gln)-neurotensin infused into the portal vein elicits the same vasoconstriction as the same dose administered i.v. indicates that the peptide is not inactivated in the liver. Instead, (Gln)-neurotensin may be activated to vasoactive substances by e.g. cleavage of the neurotensin molecule to partial sequences. Presumably such a conversion does not take place in the liver since one would then have expected a greater vasoconstriction after infusion into the portal vein than after i.v. infusion. The reason being that following i.v. infusion only about 25% of the total dose will be distributed to the liver whereas 100% of the dose will pass the liver after intraportal administration. Whether some of the partial sequences of neurotensin have vasoconstrictor properties is not known. Carraway and Leeman (1973 c) have tested partial sequences of neurotensin on several biological parameters and found that COOH-terminal sequences of 5 or more amino acids in length have full intrinsic activity. Whether formation of these peptides may occur in plasma or in some other tissue remains to be investigated.

Apart from their vasoactivity in small doses in adipose tissue and the gastrointestinal tract, neurotensins are potent inhibitors of gastric antral pressure motility and gastric acid secretion stimulated by pentagastrin or a test meal (Andersson *et al* 1976, 1977).

1976). At higher doses, neurotensins produce elevation of blood glucose (Carraway and Leeman 1973, Rosell *et al* 1976). Whether or not some or all of these effects are directly mediated by neurotensins or are due to metabolites remains to be determined.

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The effects of cholera toxin on intramural blood flow distribution and capillary hydraulic conductivity in the cat small intestine

By

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Abstract

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Blood flow distribution to the mucosa-submucosa and to the muscularis in the cat small intestine was investigated with a ^{86}Kr elimination technique before and after exposing the intestinal mucosa for 3 h to cholera enterotoxin. In all experiments the toxin induced a marked intestinal secretion. Concomitantly, intestinal blood flow was increased to a level 50 per cent above control 3 h after exposure. This reaction reflected a doubling of mean blood flow in the mucosa-submucosa while muscularis blood flow remained unchanged. In another series of experiments the effect of cholera toxin on intestinal capillary hydraulic conductivity was investigated by determining the capillary filtration coefficient (CFC). A 40% increase in CFC was noted during the 3 h observation period but this was not more pronounced than would have been expected from the concomitant vasodilatation. It is concluded that hemodynamic changes in the intestinal mucosa may be one of the several factors that probably are involved in the pathogenesis of cholera.

The cause of the profuse fluid loss in cholera is still disputed. Two fundamentally different mechanisms have been proposed (cf. Field 1974). The currently most accepted hypothesis infers that the produced fluid is the result of an increased "active" secretion and/or a decreased "active" absorption induced by the cellular action of the enterotoxin produced by the *Vibrio cholerae* organism. This hypothesis is mainly based on in vitro studies in which the involved hydrostatic, osmotic and electrochemical forces must be strictly controlled.

The other hypothesis implies that the driving force for the intestinal fluid production in cholera is the capillary hydrostatic pressure. This hypothesis was originally proposed

The term "secretion" denotes in this paper a net water transport into the intestinal lumen regardless of the underlying mechanisms.

rchow (1879) based on histopathological investigations of patients that had died of cholera. These studies suggested that the intestinal villi became denuded when exposed to the cholera vibrios. However, intestinal biopsies taken from living cholera patients demonstrated only small changes of intestinal morphology (see e.g. Gangarosa *et al.* 1960) without any signs of an intestinal villous destruction. Recent reports suggest, however, that the cholera exotoxin may increase the hydraulic conductivity of capillaries and/or of intestinal epithelium even in the absence of histological changes (see e.g. Yearley *et al.* 1973).

In order to investigate the possible involvement of circulatory parameters in the pathogenesis of the fluid losses in cholera the present study was undertaken and two types of studies were performed on the cat small bowel. In one type of experiments total blood flow and mucosal flow distribution in the gut were investigated before and after exposing the intestinal epithelium to cholera toxin. In a second type of experiments the effect of cholera toxin on the hydraulic conductivity of the intestinal capillaries was studied.

Methods

Surgical procedures and determination of total intestinal blood flow

Experiments are performed on cats anaesthetized with chloralose (30 mg/kg b. wt.) after induction with ether. The animals are deprived of food for 24 h and had no obvious signs of intestinal infection. The operative procedures used are similar to those of previous studies of intestinal circulation performed in this laboratory (Fellow *et al.* 1963, Kamp *et al.* 1964). After tracheostomy the abdomen is opened in the midline and the greater omentum and the spleen were extirpated. The experiments are carried on 15–20 cm long isolated segments from the jejunum, the remainder of the gastrointestinal tract being resected. The lumen of the segments was carefully rinsed with body-temperature saline. After hyperaemicizing the animal (3–5 mg/kg b. w.) blood pressure is measured by pressure transducer (Johns P.23 AC) in the femoral artery. The superior mesenteric vein, draining the intestinal segment of its lymph nodes, was cannulated and connected to an equal drop recorder—oscilloscope unit, recording on Grass polygraph. The venous blood was returned to the animal via the right jugular vein, the venous outflow pressure being kept around 10 mm Hg. The sympathetic nervous control is eliminated by cutting the splanchnic nerves bilaterally.

Determination of blood flow distribution

Blood flow in the mucosa-submucosa and in the muscularis was determined with ^{86}K wash-out method, using simplified modification of the technique originally described by Kamp *et al.* (1963). The radioactive isotope ^{86}K is purchased as gas from Radiochemical Centre, Amersham, England, and was dissolved in 0.9 per cent saline solution. The body-temperature solution (0.5 to 1 ml) is injected intra-arterially through bolus during 5 to 10 cm cannula in marginal intestinal artery. To record muscular blood flow the disappearance of β -radioactivity from the muscularis was recorded by means of Geiger-Müller tube under constant bias (Philips Mo 18304, diameter of window 9 mm), enclosed in lead, and placed at the submucosal border of the small intestine. The G-M tube was coupled to rate meter (Packard 4000 Series 410 A), operating on ink writer. When counts exceeded 5 000 per min, correction is made for coincidence loss using values of 100 μs for the dead time of the G-M tube. To prevent diffusion of the gas from tissue to air the intestinal surface and the submucosa was covered by Mylar. Contamination of the air with ^{86}K was reduced by continuous suction around the tracheal cannula. Counts per min were plotted at times on semilogarithmic paper. In most instances straight line was obtained but in few experiments curved curves were recorded. In these instances the straight tail part of the curve was used, the initial fast elimination rate is all probability reflecting the tracer wash out from intestinal mucosa (cf. Kamp *et al.* 1963). Intestinal blood flow (F) in ml per min and 100 g muscularis was calculated according to Kay (1951) as

$$F = k \Delta 100$$

where k denotes the rate constant of the monoexponential decay and λ the blood-tissue partition coefficient for ^{86}Kr . The latter constant was set to 1. The k value was determined according to

$$k = \frac{\ln 2}{t_{1/2}}$$

where $t_{1/2}$ denotes the half time of disappearance in min as calculated from the wash-out curve.

Blood flow in the mucosa-submucosa was calculated from the total intestinal blood flow, mucosal blood flow and the relative weights of the mucosa-submucosa. The last mentioned weight was determined for each experiment by dissecting the muscularis free from the mucosa-submucosa and weighing separately.

C. Recording of intestinal net water transport

In all ^{86}Kr wash-out experiments except one, the rate of intestinal net water transport was followed continuously with the technique described by Jodal *et al.* (1975). Briefly, the lumen of the isolated intestinal segment was perfused at a constant rate (1 ml/min) with a solution of known composition (see below). A closed perfusion system with a reservoir large enough to prevent recirculation. Changes in the perfused volume was continuously recorded by a volume transducer coupled to a Grass polygraph and connected to the perfusion system via a T tube. When no intestinal motility occurred the changes in the perfused volume reflected net water transport across the intestinal epithelium. In these experiments streptomycin (1 mg/kg b.wt.) was given to minimize motility.

D. Plethysmographic technique

To be able to estimate the hydraulic conductivity of the intestinal capillaries (i.e. their capillary filtration coefficient: CFC) the small intestine and its lymph nodes were enclosed in a triangular perspex plethysmograph according to the technique originally described by Folkow *et al.* (1963). The plethysmograph was filled with a physiological saline solution or Tyrode solution and connected to a volume transducer recording on a Grass polygraph. The temperature of the plethysmograph was maintained at 37°C continuously controlled by a thermocouple thermometer (Electrolab, Copenhagen). Polyethylene tubes inserted into the ends of the intestinal segment were fitted through openings in the plethysmograph making it possible to perfuse the lumen of the intestinal segment inside the plethysmograph. Temporarily increasing the venous outflow pressure to a known extent (usually 10 cm H_2O) evoked a characteristic Δ volume increase in 2 phases. As earlier demonstrated (Mellander 1960, Kjellmer 1965, Wallerstedt 1966) the first rapid change in volume is caused by a vascular distension, mainly of the veins, while the slow increase in volume, "the filtration slope" reflects an outward transcapillary filtration caused by the rise in capillary hydrostatic pressure.

To be able to calculate the capillary filtration coefficient (CFC) it is necessary to correlate the net outward filtration to the induced change in mean capillary hydrostatic pressure. The extent to which an increase of venous pressure is propagated to the capillary level depends on the relation between pre- and postcapillary resistances of the vascular bed (Pappenheimer 1933). To estimate this ratio in the present study a mean capillary hydrostatic pressure of 20 mm Hg was assumed. Folkow *et al.* (1963) and Lundgren and Lundgren (1972) discussed at length the error of CFC determination if the true hydrostatic capillary pressure was 10 mm Hg above or below the assumed one. It was found that the error thus introduced amounted to only 10-15 per cent, as long as the arterial blood pressure remained within the normal range (around 100 mm Hg).

E. Experimental procedures

After completing the operative procedures the animal were allowed to rest for 45-60 min. At the end of this period control measurements of the different parameters studied were made. 50-200 mg per kg b.wt. of a solution of a crude cholera toxin (freeze dried culture filtrate; NIH lot 001) dissolved in 5-10 ml physiological saline was then introduced into the intestinal lumen. The toxin was kindly supplied to us by Dr B. Holmgren, Department of Medical Microbiology, University of Göteborg. After 30 min exposure the intestinal lumen was rinsed with 50-100 ml bodywarm saline. The animal was then studied for another 30 min during which all animals included in this series developed signs of fluid loss from the intestinal epithelium.

In 4 control expts. the cholera toxin solution was heated to 56°C for 45-60 min to inactivate the toxin. This solution was then introduced into the bowel lumen as described above. In these expts. total intestinal blood flow and net water absorption rate were monitored.

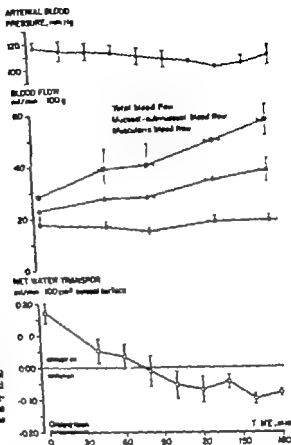


Fig. 1. Arterial blood pressure, intestinal blood flow (total and mucosal) and net water transport at six intestinal segments before and following a 30 min exposure to cholera toxin. Bars denote S.E.

Solutions

Intestines of the intestinal segments were perfused with a modified Krebs-Henseleit solution containing (mM): Na 122, KCl 4.7, KH_2PO_4 1.2, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.2, CaCl_2 2.5, mannitol 30. The osmolality of the solution ranged between 303 and 315 mOsm/kg H_2O . If necessary the osmolality of the solution was adjusted to a value within 10 mOsm/kg H_2O of that of plasma by addition of appropriate amounts of mannitol.

Results

1. Blood flow and flow distribution

Intestinal blood flow and flow distribution were studied with the ^{86}Kr elimination technique before and after a 30 min exposure of cholera toxin. The results obtained are illustrated in Fig. 1 (total and intramucosal blood flow, net water transport) and Fig. 2 (blood flow distribution). Time 0 denotes the start of the cholera toxin incubation period. Blood flows are expressed per 100 g of each individual tissue.

It is evident from Fig. 1 that the cholera toxin produced the expected change of direction of net transport of water. Approximately 60 min after the exposure to the toxin, a net fluid

where k denotes the rate constant of the monoexponential decay and λ the blood-tissue partition coefficient for ^{86}Kr . The latter constant was set to 1. The k value was determined according to

$$k = \frac{\ln 2}{t_{1/2}}$$

where $t_{1/2}$ denotes the half time of disappearance in min as calculated from the wash-out curve.

Blood flow in the mucosa-submucosa was calculated from the total intestinal blood flow, small blood flow and the relative weights of the mucosa-submucosa. The last mentioned weights were determined for each experiment by dissecting the muscularis free from the mucosa-submucosa and weighing separately.

C. Recording of intestinal net water transport

In all ^{86}Kr wash-out experiments except one, the rate of intestinal net water transport was determined continuously with the technique described by Jodal *et al.* (1975). Briefly, the lumen of the isolated small intestine segment was perfused at a constant rate (1 ml/min) with a solution of known composition (see below). A closed perfusion system with a reservoir large enough to prevent recirculation. Changes in the residual volume was continuously recorded by a volume transducer coupled to a Grass polygraph and connected to the perfusion system as a T-tube. When no intestinal motility occurred the changes in the perfused volume reflected net water transport across the intestinal epithelium. In these experiments anaesthesia (1 mg/kg b wt) was given to minimize motility.

D. Plethysmographic technique

To be able to estimate the hydraulic conductivity of the intestinal capillaries (*i.e.* their capillary filtration coefficient, CFC) the small intestine and its lymph nodes were enclosed in a triangular perspex plethysmograph according to the technique originally described by Folkow *et al.* (1963). The plethysmograph was filled with a physiological saline solution or Tyrode's solution and connected to a volume transducer recording on a Grass polygraph. The temperature of the plethysmograph was maintained at 37°C continuously controlled by a thermocouple thermometer (Electrolab Copenhagen). Polyethylene catheters inserted into the ends of the intestinal segment were fitted through openings in the plethysmograph making it possible to perfuse the lumen of the intestinal segment inside the plethysmograph. Temperature increasing the venous outflow pressure to a known extent (usually 10 cm H₂O) evoked characteristic volume increase in 2 phases. As earlier demonstrated (McLander 1960, Kjellmer 1965, Wallensten 1965) the first rapid change in volume is caused by a vascular distension, mainly of the veins, while the slower increase in volume, "the filtration slope" reflects an outward transcapillary filtration caused by the rise in postcapillary hydrostatic pressure.

To be able to calculate the capillary filtration coefficient (CFC) it is necessary to correlate the post-outward filtration to the induced change in mean capillary hydrostatic pressure. The extent to which the increase of venous pressure is propagated to the capillary level depends on the relation between pre- and postcapillary resistances of the vascular bed (Pappenheimer 1933). To estimate this ratio in the present study mean capillary hydrostatic pressure of 70 mm Hg was assumed. Folkow *et al.* (1963) and Rudfors and Lundgren (1972) discussed the length of the error of CFC determination if the true hydrostatic capillary pressure was 10 mm Hg above or below the assumed one. It was found that the error thus introduced amounted to only 10–15 per cent, as long as the arterial blood pressure remained within the normal range (around 100 mm Hg).

E. Experimental procedures

After completing the operative procedures the animals were allowed to rest for 45–60 min. At the end of this period control measurements of the different parameters studied were made. 50–200 mg per kg b wt of a solution of a crude cholera toxin (freeze dried culture filtrate; NIH lot 001) dissolved in 5–10 ml physiological saline was then introduced into the intestinal lumen. The toxin was kindly supplied to us by Dr B. Holmgren, Department of Medical Microbiology, University of Göteborg. After 30 min exposure the intestinal lumen was rinsed with 50–100 ml body temperature saline. The animal was studied for another 1–2 h during which all animals included in this series developed signs of fluid loss from the intestinal epithelium.

In 4 control expts. the cholera toxin solution was heated to 56°C for 45–60 min to inactivate the toxin. This solution was then introduced into the bowel lumen as described above. In these expts. total intestinal blood flow and net water absorption rate were monitored.

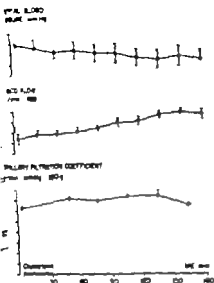


Fig. 3

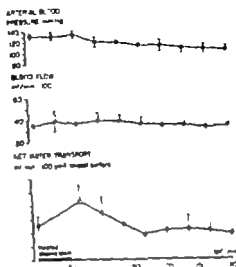


Fig. 4

Fig. 3. The effects of cholera toxin on arterial blood pressure, intestinal blood flow and intestinal capillary filtration coefficient. Bars indicate ± 3 S.E. $n=7$.

Fig. 4. The effects of heated cholera toxin on arterial blood pressure, total intestinal blood flow and net water transport. Bars indicate ± 3 S.E. $n=4$.

The submucosa constituted 10 per cent of the intestinal weight (Jodal and Lundgren 1970). Consequently, blood flows in the muscularis and in the submucosa were assumed to be identical. Table 1 reveals more clearly than Fig. 1 and 2 the redistribution occurring in the gut in response to cholera toxin.

The hydraulic conductivity of intestinal capillaries

The hydraulic conductivity of the intestinal capillaries was estimated from determinations of the capillary filtration coefficient (CFC). Such measurements were performed before and after exposing the intestinal segment to the cholera toxin. 3-5 measurements were made in the control period and the average value of these was taken as the control value. Table 3 summarizes the results obtained in 7 experiments, including the observed changes in arterial blood pressure and venous outflow. Although total intestinal blood flow increased to the extent as illustrated in Fig. 1 CFC exhibited only a slight tendency of increase. Comparing the control measurements with the last measurements in each experiment, an increase of the hydraulic conductivity was observed in all experiments, making the difference statistically significant ($p < 0.02$, Wilcoxon's matched-pairs signed-ranks test).

Control experiments

In 4 experiments the cholera toxin solution was heated to 56°C for 45-60 min prior to intestinal incubation. After such a treatment no intestinal vasodilatation or intestinal fluid loss were induced by the cholera toxin solution (Fig. 4).

BLOOD FLOW DISTRIBUTION
per cent of total blood flow

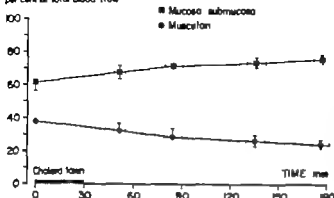


Fig. 2. Changes in blood flow distribution within the intestinal wall following 1 min exposure to cholera toxin. The curves are based on the blood flow values in Fig. 1. Bars indicate \pm S.E. $n=7$.

loss into the intestinal lumen was recorded. Concomitantly total intestinal blood flow increased. The present analyses revealed that this vasodilatation was confined to the mucosa submucosa. In this wall layer blood flow doubled during the period of observation while blood flow in the muscularis remained largely unchanged. This response pattern was also reflected in the observed changes in blood flow distribution. With increasing total intestinal blood flow an increasing fraction of total intestinal blood flow was diverted to the mucosa submucosa (Fig. 2).

In 4 experiments, blood flow in the mesenteric lymph nodes was studied before and 3 h after exposing an intestinal segment to cholera toxin. Blood flow in the lymph nodes was determined after gently occluding the vessels supplying the intestine during short periods. An increased lymph node flow was recorded after cholera toxin but it was of approximately the same relative magnitude as the one observed for total blood flow. Hence, the increase in total blood flow illustrated in Fig. 1 cannot be explained by an exaggerated vasodilatation confined only to the lymph nodes.

A further analysis of the blood flow redistribution occurring after exposure to cholera toxin is presented in Table I. In this Table the blood flow and flow distribution to each individual layer has been calculated making two assumptions. First, it was assumed that

TABLE I. Blood flow and flow distribution before ("no toxin") and 3 h after exposing the small bowel to cholera toxin ("cholera"). Mean \pm S.E. $n=7$

	Control	Cholera
Total blood flow ml/min 100 g	43 \pm 2	36 \pm 4
<i>Regional blood flow ml/min \times 100 g</i>		
mucosa	3 \pm 4	65 \pm 7
submucosa	18 \pm 2	17 \pm 2
muscularis	18 \pm 2	17 \pm 2
<i>Blood flow distribution, per cent</i>		
mucosa	54 \pm 6	71 \pm 3
submucosa	8 \pm 1	3 \pm 1
muscularis	38 \pm 5	24 \pm 3

ood, a number of histological observations seem to indicate an increased capillary permeability in cholera. Thus, increases of capillary permeability to colloidal iron-dextran w above 190 000; Keusch *et al* 1967), ferritin (m.w. around 346 000- Daifdorf *et al* 1971) and horseradish peroxidase (m.w. 40 000- Yardley *et al* 1973). Furthermore, most morphological studies indicate the presence of a tissue edema in cholera located to the core and the submucosa (Gangarosa *et al* 1960) and often wide "dilated" capillaries in the villi (Yardley *et al*, 1973).

However in the present study no experimental support for a substantially increased hydraulic conductivity of the intestinal capillaries in cholera was obtained, to judge from only slightly increased capillary filtration coefficients (CFC) in the face of a fairly marked mesal vasodilatation. Inducing a similar blood flow increase by a vasodilator drug, with effect on capillary permeability clearly increased CFC (Folkow *et al*, 1963). The tracers used in the studies cited above were all of high molecular weight, passing the capillary lumen through "leaks" (Grotte 1956) and/or by pinocytosis (Carter *et al* 1974). The fluid transfer through leaks and by pinocytosis is in all probability contributing very little to overall capillary hydraulic conductivity (Grotte 1956). The present results may therefore be explained by assuming that the cholera toxin only increased capillary permeability to small molecules by increasing the size of occasional capillary "leaks" and/or augment pinocytosis while the conventional pores of the fenestrated mucosal capillaries were not affected by the toxin. An alternative explanation may lie in that a change of capillary permeability was confined only to a small portion of all intestinal capillaries.

In this context it should be underlined, however that the absence of any direct effect of cholera toxin (m.w. 81 000- Finkelstein and LoSpalluto 1972) on capillary permeability in this study may be due to the relative impermeability of the intestinal epithelium to such large polar solutes as the toxin. The increase of vascular permeability demonstrated in the experiments cited above, may therefore not be relevant with regard to the situation in the intestinal wall.

Mean capillary hydrostatic pressure in cholera. The tissue edema often reported in the cholera intestinal wall (see above) may not necessarily reflect an increased capillary permeability but may be the result of an increased mean capillary hydrostatic pressure (Folkow *et al*, 1963). The results of the present study make it possible to calculate the increase of capillary pressure necessary to produce the rate of secretion observed in this study about 3 h after the exposure to the cholera toxin. Assuming that the capillary hydraulic conductivity of the capillaries in the muscularis and in the submucosa is the same as in skeletal muscle, i.e. 1/10 of that of the whole intestine (cf. Cobbold *et al* 1963 Folkow *et al* 1963), the hydraulic conductivity of the intestinal mucosal capillaries can be calculated. In this study one then derives a mucosal capillary filtration coefficient of 0.15 ml/min mmHg 100 g mucosal tissue. Mean absorption rate in the control situation amounted to 1.11 ml/min 100 g mucosa and mean secretory rate at 3 h after cholera exposure was about 0.52 ml/min 100 g mucosal tissue. Hence, an increase of P of 11 mmHg ($(1.11 + 0.52) / 0.15$) would produce the secretory rate observed in the feline gut of this study after cholera toxin exposure, assuming that all the filtered fluid reached the intestinal lumen. This is not probable for several reasons, one being that filtered fluid is also transported towards the "inside" of the

Discussion

The present study has revealed that the incubation of cholera toxin in the skin produced within 1–2 h a secretory state, as also reported by Finck and Katz (1972). Dlugolecka (1974). Quantitatively the secretory rate was the same as the highest intestinal absorptive rate observed in the cat. In man that would be equivalent to a secretion of 400 ml/h. Furthermore, the net secretion of fluid induced by the cholera toxin was accompanied by an increase of total intestinal blood flow. An analysis of the local blood flow distribution revealed that the vasodilatation occurred exclusively in the mucosa. In the submucosa where blood flow was doubled 3 h after exposing the intestine to the toxin the hydraulic conductivity of the intestinal capillaries was, however, only slightly increased. The toxin despite the induced, quite substantial mucosal vasodilatation.

Intestinal blood flow in cholera With regard to the effects of cholera toxin on intestinal hemodynamics, two earlier studies have been published by Carpenter *et al.* (1968) and Norris and Summer (1974). Carpenter and coworkers reported on an average 50% increase of superior mesenteric artery blood flow in the dog 1 h after the administration of the cholera toxin to the stomach, i.e. a vasodilatation of the same magnitude as reported here (Fig. 1 and 3).

Norris and Summer (1974) on the other hand, neither recorded any blood flow increase upon exposing the intestine to the cholera toxin, nor any significant change of local blood flow distribution. In their investigation a technique was used that in principle is similar to the present one. However Norris and Summer made a compartmental analysis of the wash-out of γ -activity recorded after La injection of ^{133}Xe , as was done by Kety *et al.* (1968). Our reason for not using this approach was that one cannot, without extensive control experiments, assume that the different components of a multiple desaturation curve reflect the same biological events in two such different experimental conditions as the normal and the choleraic intestines. As no such control studies were performed, the results by Norris and Summer are difficult to evaluate.

The mechanism underlying the cholera-induced vasodilatation is not known. It is improbable that products from the villous cells are responsible for the hyperemia. In the great majority of vascular smooth muscles controlling mucosal blood flow are located in the vessels situated in the deeper mucosal parts. Recently however a high concentration of vasoactive intestinal peptide (VIP) was demonstrated in the lumen of choleraic intestine (Bloom *et al.* 1977). VIP is a "candidate hormone" in the gut (Grossman 1974) that has been proposed to be a neurotransmitter. This may suggest that the cholera toxin induces the release of certain hormones in the crypts that may exert a local effect on the vessels and/or the toxin elicits an intramural nervous reflex.

Capillary permeability in cholera Two types of experiments seem to suggest that cholera toxin increase capillary permeability which, in turn, may contribute to the pathogenesis of cholera. First, injecting the toxin into the skin of guinea pigs or rabbits (Craig 1965; Finkelstein *et al.* 1966) produced a local tissue edema indicative of an effect on vascular permeability. This effect could not be dissociated from the effects of the cholera toxin even when using the most purified preparations of the toxin (Finkelstein and Lo Spalluto

re absorption. However Lee and Silverberg (1972) and Strombeck (1972) have obtained results which seem incompatible with this view by demonstrating that intestines in dog and rat, which during *in vivo* conditions secreted fluid after exposure to cholera toxin, are capable of fluid absorption during *in vitro* conditions. Thus, most probably the intestinal fluid loss in cholera is the result of several mechanisms, increased capillary filtration being one. Furthermore, we are currently investigating the effects on the cholera toxin on the countercurrent multiplication of sodium chloride in the villi. The villous osmolarity created by this mechanism is probably of great importance for the intestinal reabsorption of water (Jodal *et al.* 1978). Preliminary experiments suggest that exposing the rat to cholera toxin markedly diminishes tissue osmolarity in the villi, thus further enlarging the multifactorial background to the cholera secretion.

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mucosa (i.e. the villous core and the lymphatics) and another being that all microcapillaries may not be adjacent to the lining epithelium. These calculations clearly suggest that the hydraulic conductivity of the intestinal mucosal capillaries is high enough to account for large filtration rates at comparatively small changes in P_c .

The question then arises: did any hemodynamic changes occur in the gut after exposure to cholera toxin which may produce an increased P_c ? One may then recall that blood flow doubled in the mucosa, probably mainly due to a vasodilatation at the precapillary level. Hence, a marked increase of P may have been induced. Unfortunately with the present method it is not possible to determine the pre- to postcapillary resistance ratios. However, it has been demonstrated in skeletal muscle that a doubling of blood flow by vasodilatation augments tissue volume at a rate corresponding to an increase of P of about 5 mmHg (Kjellmer 1964).

The discussion above has been carried out assuming that the intestinal epithelium does not offer any resistance to transepithelial fluid transport. However this assumption is probably not true since most epithelia are considered to have a hydraulic conductivity well below that of capillaries. In fact for a long time most epithelia were considered to be perfectly tight, only allowing fluid transport of water through cellular "pores". However, evidence has lately accumulated that epithelia may be quite leaky, particularly when pressure is applied to the "tissue" side, at least during *in vitro* conditions. For example, Halperin & Lifson (1969) obtained a value of the epithelial hydraulic conductivity which was 10-100 times larger than that of the capillary permeability when expressed per unit area. A comparable high leakiness has also been observed in similar experimental conditions in other epithelia (for ref. see House 1974). Moreover, according to some authors, epithelial permeability is increased in cholera (see e.g. Love 1969).

To summarize: earlier reports and the present study have provided experimental support for the view that increased capillary permeability (mainly for large molecules) and increased mean capillary pressure in the mucosa may contribute to the intestinal fluid loss seen in cholera, although it cannot be considered to be proven. In reviews these proposals are dismissed with reference to the results obtained by Carpenter *et al.* (1969). This study is the only one in which a direct attempt has been made to investigate the possible involvement of capillary forces in cholera. Dogs were infected with cholera vibrios and during the acute phase of the disease arterial blood pressure was lowered for periods of 90 min. By lowering arterial blood pressure to 50 mm Hg or below did not reduce the rate of cholera fluid loss. However, these results do not exclude the involvement of capillary forces in cholera for at least two reasons. First, even pronounced changes of arterial blood pressure affects P very little by proper adjustment of the pre- to postcapillary resistance ratio, due to the myogenic autoregulatory capacity of the intestinal vascular bed (Haglund & Lundgren 1972, Gore and Bohlen 1975). Second, the mere lowering of arterial blood pressure to 50 mm Hg or below for 90 min may in itself produce a net fluid loss into the intestinal lumen also in most normal animals (Haglund and Lundgren 1972). Such a fluid loss cannot be distinguished from the cholera diarrhea.

As indicated in the introduction the currently most accepted hypothesis infers that the fluid loss in cholera reflects an exaggerated "active" intestinal secretion and/or an inhibition

re absorption. However Lee and Silverberg (1972) and Strombeck (1977) have noted results which seem incompatible with this view by demonstrating that intestines of dog and rat, which during *in vivo* conditions secreted fluid after exposure to cholera toxin, are capable of fluid absorption during *in vitro* conditions. Thus, most probably the intestinal fluid loss in cholera is the result of several mechanisms, increased capillary secretion being one. Furthermore, we are currently investigating the effects of the cholera toxin on the countercurrent multiplication of sodium chloride in the villi. The villous osmolarity created by this mechanism is probably of great importance for the intestinal reabsorption of water (Jodal *et al.* 1978). Preliminary experiments suggest that exposing the gut to cholera toxin markedly diminishes tissue osmolarity in the villi, thus further explaining the multifactorial background to the cholera secretion.

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Aspects on diamine oxidase activity and its determination

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Abstract

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Homogenates of guinea-pig liver and human placenta, tissues known to be rich in diamine oxidase, were incubated with ^{14}C -putrescine and the metabolites formed were determined. In the incubate of guinea-pig the major metabolites were GABA and some unidentified compound(s). Δ -pyrroline and $^{14}\text{CO}_2$ were not obtained. In both the maternal and fetal parts of human placenta, radioactive GABA and the unidentified compound(s) as well as Δ -pyrroline were found. The results indicate that GABA is an important metabolite in putrescine metabolism. Determination of the amount of Δ -pyrroline is thus not a satisfactory measure of the diamine oxidase activity in tissues.

Key words: Diamine oxidase activity, putrescine metabolism, GABA, Δ -pyrroline

Diamine oxidase (EC 1.4.3.6) catalyses the oxidation of diamines such as histamine, putrescine and cadaverine, yielding in the process an aminoaldehyde. The enzyme was thought to attack principally histamine and for that reason was named histaminase. It was later found that putrescine and cadaverine are the preferred substrates (for ref. see Zeller 1965; deley *et al.* 1970).

It is well established that when putrescine is incubated in the presence of purified diamine oxidase γ -aminobutyraldehyde is formed which almost quantitatively is transformed into the cyclic compound Δ -pyrroline. The *in vitro* measurement of diamine oxidase activity owing to Okuyama and Kobayashi (1961) is in reality based upon the determination of Δ -pyrroline formed from added ^{14}C -putrescine to the incubate. The Δ -pyrroline is added into a toluene liquid scintillation mixture for assay of the radioactivity. This method is widely used, mostly for measurement of diamine oxidase activity in plasma. The method is also used in the determination of diamine oxidase activity in tissues where the method has not been well characterized.

It has been shown that injection of ^{14}C -putrescine was followed by the expiring of large amounts of radioactive CO_2 within a couple of hours and γ -aminobutyric acid (GABA) was found to be an intermediate in the catabolism of putrescine to carbon dioxide (Seiler and

Eichentopf 1975 Henningsson and Rosengren 1976) The catabolism of ^3H -putrescine, radioactive GABA and CO_2 was blocked by aminoguanidine, a specific diamine oxidase inhibitor

The aim of the present study was to determine the nature of the metabolic products from putrescine via the diamine oxidase pathway on incubating tissues *in vitro* and to develop another approach to the assay of diamine oxidase activity in tissues taking into consideration the main products formed in the incubate, thus applying our *in vivo* findings of putrescine catabolism on *in vitro* studies. A preliminary report of this work has been presented elsewhere (Andersson *et al.* 1977)

Methods

Adult female guinea-pigs, weight 830–950 g, were used. They were fed a standard pellet diet and had *ad libitum*. The animals were stunned and exsanguinated whereafter pieces from the liver lobes were removed immediately. The tissue was cooled on ice, minced with scissors, weighed and gently homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 4 volumes of 0.1 M cold sodium phosphate buffer, pH 7.2 containing 0.2% (w/v) glucose. This homogenate was used as enzyme source.

Human placenta was obtained at term and was divided into its maternal and fetal parts. The maternal part was taken from the outermost layer and the fetal part from a layer III depth of about 1 cm from the surface. Homogenate was prepared in the same way as above.

Incubation procedure The incubation mixture contained homogenate corresponding to 50, 80 or 100 mg of tissue, 1.4×10^{-4} M putrescine (final conc. 10^{-4} M, sp. act. 0.1 ± 0.5 mCi/mmol) and the same buffer as used for homogenization, the total finally made up to a volume of 1.5 ml. The mixture was incubated for 1–4 times at 37°C . Blanks were provided by preincubation with 100 μl aminoguanidine hemisulphate (final conc. 2×10^{-4} M). The incubation was stopped by tipping 0.7 ml 2 M hydrochloric acid (samples subjected to thin-layer electrophoresis) or 0.7 ml 12.6% (w/v) sulfosalicylic acid (samples subjected to reversed-phase liquid chromatography) into the incubate from a side arm of the incubation vessel. A further 45 min standing allowed for complete absorption of expelled ^3H - CO_2 (see below).

Determination of radioactive substances resulting from catabolism of ^3H -putrescine The following putrescine metabolites were determined: CO_2 , GABA, Δ^1 -pyrrolidine and, in addition, some unknown compound(s). Each of these products (with the exception of ^3H - CO_2) was measured in a different way. Some incubates were used for assays by reversed-phase liquid chromatography. Parallel incubates were divided into two parts, where one was used for extraction into diethyl ether, the other for thin-layer electrophoresis. Duplicates were performed of all determinations with the exception for assays carried out with reversed-phase liquid chromatography. Thin-layer electrophoresis and automated liquid chromatography were used for determination of the amount of nonradioactively labelled putrescine at the end of incubation. 1. Expelled ^3H - CO_2 was trapped on a 10 \times 25 mm piece of No. 005 Munktell's filter paper prepared with 100 μl hydroxide of Hyamine 10-X. After termination of the incubation the filter paper was dropped into 5 ml of Bray scintillation mixture (Bray 1960) and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB, 2002).

2. Chromatographic separation of GABA was carried out using an automatic amino acid analyzer (Beckman BIOCAL 3201). Application of liquid chromatography to diamine research has been described by Knecht *et al.* (1970). The procedure as carried out in our laboratory is as follows. After centrifugation and adjustment of pH to 2.0–2.5 with N-OH the incubate was filtered through a filter with a pore size of $0.2 \mu\text{m}$ (Whatman, Bedford, Mass. 01730). The radioactive compounds of the incubate were separated at 60°C on a column of Durrum DC 6A. In separating amino acids and amines, four buffers with different pH and different amounts of sodium citrate and sodium chloride were pumped through the column in sequence at a flow rate of 80 ml/h for different elution times. Buff. A: 0.20 M Na $^+$ pH 3.90, 44 min. Buff. B: 0.35 M Na $^+$ pH 4.44 min. Buff. C: 0.90 M Na $^+$ pH 6.20, 47 min. Buff. D: 0.80 M Na $^+$ pH 5.90, 40 min. After passage through the column the stream was split according to Lou (1973). One half proceeded to a fraction collector by which 2 ml fractions could be collected throughout the analysis. Of these fractions about 1 ml was added to 3 ml Instagel (Packard) scintillation solution and the radioactivity was measured. The other half of the column eluate was mixed with ninhydrin and peak were determined by ninhydrin.

as in the regular way. The final identification of the compounds in relevant fractions of eluates from amino acid analyzer has been described (Hestdalsson and Rosenberg 1976).
 addition radioactive GABA as estimated by thin-layer electrophoresis. Aliquots of the incubate centrifuged at 80 g for 20 min. Of the supernatant 250 μ l as added to 5 μ l of standard carrier solution (1 μ g unlabelled GABA and 1 μ g unlabelled putrescine in 5 μ l) and 2.5 ml ethanol completed the mixture of proteins. The samples are stored at 11°C until electrophoresis was performed. Next, mixture is centrifuged, the supernatant as evaporated to dryness, the residue as resolved in 2.5 μ l 0% ethanol and quantitatively applied to thin-layer plate (TLC plates silica gel 60, Merck). The thin-layer electrophoresis each separated the radioactive compounds was performed at 20 V/cm for 70 min and at 3°C using pyridine-acetic acid buffer pH 4.8 (Fraser and Bolen 1957). The plate as dried and the relevant fractions were removed in spots. The silica gel powder was transferred to vials containing 10 ml elution solution (3.5 g Permabond III, Packard, in one litre of toluene) and the radioactivity as stored.

The procedure employed for determination of Δ^1 -pyrroline as the method derived by Okiyama and Watanabe (1961). Knecht et al (1973) has given detailed description of the procedure. Aliquots of the eluate were mixed with saturated NaHCO_3 and NaOH solution to obtain pH of 9.5-10.1. 1 ml of sample was transferred to test tube containing 4 ml cold toluene. The test tube, equipped in glass vial, as vigorously shaken for 4 min, centrifuged at 900 g for 1 min after which the aqueous phase was broken at 20 to 30°C in bath of ethanol and solid carbon dioxide. Four ml of the upper toluene phase was transferred to vials containing 4 ml scintillation solution (11 g Permabond III Packard, in one litre of toluene). The radioactivity was monitored.

For determination of radioactive Δ^1 -pyrroline was also carried out by the automatic amino acid analyzer described above. In identifying Δ^1 -pyrroline, standard test solution was prepared by incubating ^{14}C -putrescine with purified diamine oxidase (Boehringer Grade II, Sigma).

Incubate unlabelled radioactive compound(s) appeared in the samples and as determined by either of the two methods, i.e. the automated liquid chromatography or the thin-layer electrophoresis.

Results

In initial experiments it was shown that on incubating ^{14}C -putrescine with crude liver homogenate $^{14}\text{CO}_2$ as well as ^{14}C - Δ^1 -pyrroline were formed while at incubation with the supernatant of the liver homogenate only pyrroline was formed (Table I). These expts. also showed that the catabolism of putrescine was considerably higher in the homogenate than in the supernatant. The results encouraged the search for intermediate metabolites of radioactive putrescine. In these expts. homogenate of guinea-pig liver was incubated with ^{14}C -putrescine 1 h. The putrescine metabolites, as well as the residue of added putrescine, were determined in either of two systems. The results are seen in Table II. Except for the unknown compound(s), a good agreement was obtained by the methods used. The discrepancy regarding the unknown compound(s) is the subject of further investigation. Using the automated liquid chromatographic method a split-stream technique is necessary to determine radioactive compounds, a technique which is rather expensive and laborious. For that reason henceforth in this study refer to results obtained by the toluene extraction of pyrroline and by thin-layer electrophoresis of the other putrescine derivatives.

From Table II it appears that an inverse relation existed between on the one hand the amount of unmetabolized putrescine and, on the other the amount of GABA and the unknown compound(s), i.e. the more substrate used up the more GABA and unknown compound(s) were formed. Regarding pyrroline no such relation was found.

Experiments were performed to demonstrate the relationship between the rate of formation of the different putrescine metabolites and the amount of tissue in the incubate. Aliquots

Elchentopf 1975 Henningsson and Rosengren 1976) The catabolism of ^3C -putrescine radioactive GABA and CO was blocked by aminoguanidine, a specific diamine α -inhibitor

The aim of the present study was to determine the nature of the metabolic products from putrescine via the diamine oxidase pathway on incubating tissues *in vitro* and to develop another approach to the assay of diamine oxidase activity in tissues taking into consideration the main products formed in the incubate, thus applying our *in vivo* findings of putrescine catabolism on *in vitro* studies. A preliminary report of this work has been presented elsewhere (Andersson *et al* 1977)

Methods

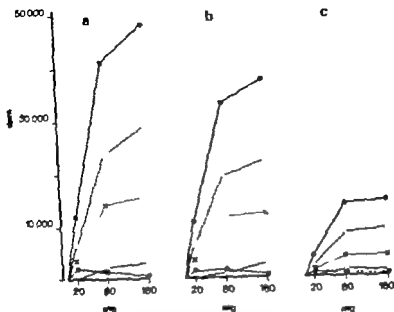
Adult female guinea-pigs, weight 830–930 g, were used. They were fed a standard pellet diet and *ad libitum*. The animals were stunned and exsanguinated whereafter pieces from the liver lobes were removed immediately. The tissue was cooled on ice, minced with scissors, weighed and gently homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 4 volumes of 0.1 M cold sodium phosphate buffer, pH 7.2 containing 0.1% (w/v) glucose. The homogenate was used as enzyme source.

Human placenta was obtained at term and was divided into the maternal and fetal parts. The maternal part was taken from the outermost layer and the fetal part from a layer at a depth of about 1 cm from the surface. Homogenate was prepared in the same way as above.

Incubation procedure The incubation mixture contained homogenate corresponding to 20, 80 or 160 mg of tissue, 1.4×10^{-4} M putrescine (final conc. 10^{-4} M; sp. act. 0.125 mCi/ μmol) and the same buffer as used for homogenization, the total finally made up to a volume of 1.5 ml. The mixture was incubated for 0, 1, 2, 4, 8 and 16 times at 37°C. Blanks were provided by preincubation with 100 μl aminoguanidine hemisulphate (final conc. 2×10^{-3} M). The incubation was stopped by tipping 0.7 ml 2 M hydrochloric acid (samples were then subjected to thin-layer electrophoresis) or 0.7 ml 12.6% (w/v) sulfolactic acid (samples were then subjected to liquid chromatography) into the incubate from a side arm of the incubation vessel. A further 45 sec was allowed for complete absorption of expelled $^3\text{CO}_2$ (see below).

Determination of radioactive substances resulting from catabolism of ^3C -putrescine The following putrescine metabolites were determined: CO_2 , GABA, Δ^1 -pyrroline and, in addition, some unidentified compound(s). Each of these products (with the exception of $^3\text{CO}_2$) was measured in two different ways. Some incubates were used for assays by automated liquid chromatography. Parallel incubates were divided into two parts, where one was used for extraction into toluene, the other for thin-layer electrophoresis. Duplicates were performed of all determinations with the exception for assays carried out with automated liquid chromatography. Thin-layer electrophoresis and a reversed liquid chromatography were also used for determination of the amount of unmetabolized labelled putrescine at the end of incubation. 1. Expelled $^3\text{CO}_2$ was trapped on 10–25 mm pieces of No. 005 M Whatman's filter paper pre-equilibrated with 100 μl hydroxide of Hyamine 10-X. After termination of the incubation the filter paper was dropped into 8 ml of Bray's scintillation mixture (Bray 1960) and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB 200.).

2. Chromatographic separation of GABA was carried out using an automatic amino acid analyzer (Beckman BIOCAL 3201). Application of liquid chromatography in diamine research has been described by Perle *et al.* (1970). The procedure as carried out in our laboratory is as follows. After centrifugation and adjustment of pH to 2.0–2.5 with NaOH the incubate was filtered through a filter with pore size of 0.2 μm (Whatman Corp., Bedford, Mass. 01730). The radioactive compound of the incubate were separated at 60°C on a column of Durram DC 6A. I separation amino acid and amines, four buffers with different pH and amounts of sodium citrate and sodium chloride were pumped through the column in sequence at a flow rate of 80 ml/h for different elution times: Buff. A: 0.20 M Na⁺ pH 3.50, 44 min; Buff. B: 0.35 M Na⁺ pH 4.44 min; Buff. C: 0.90 M Na⁺ pH 6.20 47 min; Buff. D: 2.80 M Na⁺ pH 5.90, 40 min. After passing the column the stream was split according to Lou (1973). One half proceeded to a fraction collector by which 2 ml fractions could be collected throughout the analysis. Of these fractions an aliquot of 0.5 ml was added to 3 ml Instagel (Packard) scintillation solution and the radioactivity was measured. The other half of the column eluate was mixed with ninhydrin and peaks were detected by ninhydrin.



1. Rate of formation of different metabolites from ^{14}C -putrescine in guinea-pig liver as related to the mass of tissue. The metabolites are CO_2 (○—○), Δ^4 -pyrroline (●—●), GABA (□—□) and unknown compound(s) (■—■). The total amount of these metabolites is also given (●—●).

not with 160 mg, this applicable to both the expts. with high and low oxidative deamination.

The relationship between the incubation time and amount of products formed was also studied (Fig. 2). The tissue amount in the samples was 20 mg in 2 expts. and 80 mg in two others. GABA and the unknown compound(s) were again the major metabolites and the production of them was linear up to at least 1 h. With 80 mg of tissue there was a linear relation between the amount of $^{14}\text{CO}_2$ produced and the incubation time. With 20 mg of tissue the production of $^{14}\text{CO}_2$ also rose linearly with increasing incubation time, but it was slightly small. The time curve for Δ^4 -pyrroline showed a progressively increasing course up to 120 min in samples with 20 mg tissue. However in samples with 80 mg tissue the amount of Δ^4 -pyrroline increased up to 60 min whereafter there was a decline in the formation of Δ^4 -pyrroline. The total amount of putrescine metabolites found plotted against time showed a straight line up to 2 h in expts. with 20 mg tissue in the incubate. With 80 mg the curve was linear to only 60 min.

The oxidative deamination of ^{14}C -putrescine on incubating human placenta was also studied. 80 mg of tissue was incubated for 30 and 90 min. The degradation of ^{14}C -putrescine was approximately 10 times higher in the maternal part than in the fetal one. In both parts inactive GABA and the unknown compound(s) were formed as well as Δ^4 -pyrroline. Formation of $^{14}\text{CO}_2$ could also be recorded. In contrast to the expts. with guinea-pig liver Δ^4 -pyrroline was the major putrescine derivative. In the maternal part the amount of pyrro-

TABLE I Formation (nmol/g h) of radioactive Δ^1 -pyrroline and CO_2 from ^{14}C -putrescine in homogenate (H) and supernatant (S) of guinea-pig liver in 3 experiments. The sum of the two products given.

Δ^1 -pyrroline		CO		Δ^1 -pyrroline plus CO_2	
H	S	H	S	H	S
84	77	410	0.255	490	78
51	30	450	0.52	500	31
61	24.2	350	7.6	410	32

of homogenate corresponding to 20, 80 and 160 mg of liver from the same animal incubated for 1 h. The following products of ^{14}C -putrescine were noted: CO_2 , Δ^1 -pyrroline, GABA and the unknown compound(s). The major metabolites formed were GABA and unidentified compound(s) (Fig. 1). In the three experiments referred to in Fig. 1 there is a linear relationship between tissue amount and rate of formation of these two metabolites up to 80 mg tissue per sample. With 160 mg of tissue the curve was flattening in all 3 experiments. As to $^{14}\text{CO}_2$, the production of this putrescine metabolite was low and an approximately linear relation to the tissue amount was found; this is in contrast to Δ^1 -pyrroline, the production of which was highest in the samples with low tissue amount. In Fig. 1 the total amount of the observed putrescine metabolites is also plotted. In 2 of the 3 experiments the overall decarboxylation was high: the sum of the products constituting 21 and 18% of the ^{14}C -putrescine substrate in samples of 20 mg, and 83 and 64%, respectively, in samples of 160 mg (Fig. 1a and 1b). The third liver had a lower enzyme activity: the products constituting 7% in samples with homogenate of 20 mg tissue and 24% with 160 mg (Fig. 1c). The curve representing the sum of putrescine metabolites is approximately linear with the tissue amount of 20 and 80

TABLE II Comparison of different methods used for the assay of metabolic products [Δ^1 -pyrroline, GABA and an unknown compound(s)] of ^{14}C -putrescine and the residue of putrescine after isolation of ^{14}C -putrescine (38 000 dpm/sample) with liver homogenate. The methods used were: reversed-phase liquid chromatography (ALC), toluene extraction (TE) and thin-layer electrophoresis (TLE). The figures are dpm/sample.

	Δ^1 -pyrroline		GABA		Unknown compound(s)		Putrescine	
	ALC	TE	ALC	TLE	ALC	TLE	ALC	TLE
	1460	950	4000	4500	3200	330	45000	47000
	248	410	8900	15600	5300	5900	—	29300
	1630	1990	5000	6400	3500	3500	38000	45000
	1460	1350	27100	23400	20400	14100	5300	8500
	810	540	31000	28800	23100	15500	3000	5900
	1410	1370	6700	5900	5600	3300	48000	46000
	1610	1430	18900	19100	20700	11500	17700	19700
	284	540	19900	21900	15600	12000	18800	16600
	1110	1070	15100	15700	12100	8500	25700	27200
SE								
\pm S.E.M.	± 206.0	± 194.9	± 3720	± 340	± 3020	± 1880	± 7000	± 6000

III. Formation of CO_2 , Δ^1 -pyrroline, GABA and the unknown compound(s) from ^{14}C -putrescine in fetal human placenta. Aliquots of placental tissue homogenates were incubated for 30 and 90 min. The rates of the products is also given. The figures are given, example.

	Δ^1 -pyrroline		GABA		Unknown compound(s)		Sum	
	30 min	90 min	30 min	90 min	30 min	90 min	30 min	90 min
32	2 600	4 800	510	2 140	0	1 990	3 200	8 900
16.7	510	1 300	400	2 380	330	1 750	1 440	5 800
33	1 640	4 900	300	1 010	310	1 290	2 250	9 200
35	2 170	5 490	2 580	3 500	100	1 570	4 900	10 500
—	12 700	—	5 700	—	1 330	—	19 800	—
—	1 220	—	700	—	640	—	2 560	—

is known that γ -aminobutyraldehyde is non-enzymatically converted into Δ^1 -pyrroline that at steady-state the two compounds exist in equilibrium (Jakoby and Fredericks 7). The method elaborated by Okuyama and Kobayashi (1961) measures only one of the facts formed from putrescine, *Le* Δ^1 -pyrroline. In the present study the amount of roline was found unrelated to the amount of tissue and the time of incubation. In addition he guinea-pig liver the pyrroline represented only a small fraction of the products formed in putrescine via the diamine oxidase pathway. It thus appears that the method of Okuyama and Kobayashi (1961) should not be used indiscriminately for determination of diamine oxidase activity in tissues. This rapid method has been widely used, mostly for determination diamine oxidase activity in plasma. It has also been used for assay of diamine oxidase activity in tissues utilizing homogenate or supernatant of tissue extract. For determination diamine oxidase activity in plasma the method appears safe as plasma lacks enzymes which degrade γ -aminobutyraldehyde.

In the guinea-pig liver the major radioactive metabolite of putrescine found, besides ABA, was an unidentified compound(s). This is in agreement with our results after administration of ^{14}C -putrescine to mice (Hemmingson and Rosengren 1976). Seiler and Eichenhoff (1975) have observed urinary excretory products of putrescine, which they suggest are unknown reaction products of the highly reactive γ -aminobutyraldehyde or of Δ^1 -pyrroline. The unknown compound(s) observed in our study was not extractable in solvents and is not found when ^{14}C -putrescine was incubated with purified hog kidney diamine oxidase. These facts together with the observation that the production of the unknown compound(s) is approximately proportional to the tissue amount and the time of incubation suggest that the unknown metabolite(s) is formed enzymatically. In preliminary expts. formation of the unknown compound(s) on incubating homogenate of guinea-pig liver with ^{14}C -GABA is negligible in spite of a high formation of $^{14}\text{CO}_2$ indicating the unknown compound(s) is not being a metabolic product of GABA.

The production of GABA in relation to Δ^1 -pyrroline was lower in the human placenta than in guinea-pig liver. The reason for this is so far not known. One possibility is that the relation between diamine oxidase and γ -aminobutyraldehyde dehydrogenase may be different in the two tissues. Alternatively the dehydrogenase might be a more labile enzyme than dia-

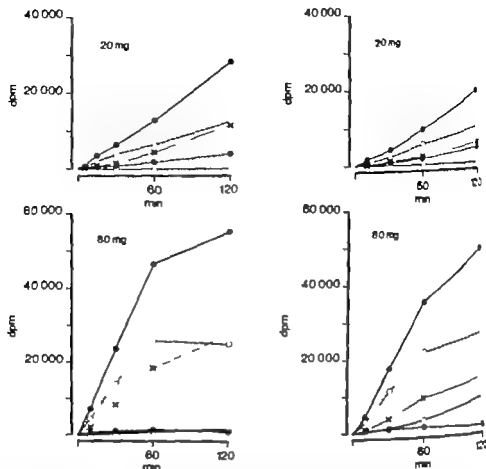


Fig. 2. Rate of formation of different metabolites from ^{14}C -putrescine in guinea-pig liver as related to time of incubation. The metabolites were CO_2 (O—O), Δ^2 -pyrroline (●—●), GABA (O—O) and known compound(s) (x—x). The total amount of these metabolites is also given (●—●).

line formed in the samples was so high as to interfere with the determination of the products in the thin-layer electrophoresis system. Results obtained on incubating liver fetal placenta are given in Table III.

Discussion

The present *in vitro* study shows that mammalian tissues rich in diamine oxidase are capable of producing various compounds derived from putrescine—compounds which recently have been found after *in vivo* administration of putrescine (Sæller *et al.* 1971; Henningsen & Rosengren 1976). Our present work also indicates that diamine oxidase is important in putrescine metabolism, one of the major metabolites formed being GABA. *In vivo* studies with ^{14}C -GABA have shown it to enter the tricarboxylic acid cycle (Roberts 1956; Wieth *et al.* 1959). Thus, a main route in the metabolism of putrescine seems to be a multi-step sequence with several enzymes involved where the first two steps of the route are catalysed by diamine oxidase and γ -aminobutyraldehyde dehydrogenase respectively. In connection with the production of CO_2 as related to time of incubation, the multi-step sequence in the way between putrescine and CO_2 should be kept in mind.

Tension-length behaviour of a molluscan smooth muscle related to filament organisation

By

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Abstract

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Under optimal activation conditions, we obtain an active tension-length curve with well-defined plateau and trough. The plateau can be explained in terms of sliding filament mechanisms in which double overlap of thin filaments does not interfere with cross-bridge formation between thick and thin filaments of the opposite polarity.

The shape of the tension-length curve in vertebrate skeletal muscle can be explained on the sliding filament theory according to which the tension developed is proportional to the amount of overlap between the (thin) actin and (thick) myosin-containing filaments (Huxley and Niedergerke 1954, Huxley and Hanson 1954). To provide a quantitative demonstration of this relation it is necessary to know the length of the thin and thick filaments, and to have a method of relating the tension-length curve to filament overlap (Gordon, Huxley and Allen 1966).

At first sight there would seem to be little chance of accounting for the tension-length curve of a smooth muscle in terms of filament overlap. In the absence of a visible band structure there is no easy way of relating overlap to muscle length. Furthermore it is difficult to interpret the extent to which the behaviour of a whole muscle reflects the mechanical properties of its individual cells. Experiments with single smooth muscle cells have only been attempted very recently and still present formidable difficulties. Another serious obstacle is that most smooth muscles respond to stretching by spontaneous contractile activity (Pomeroy 1974). On the structural side estimates for the length of thin and thick filaments are so far available only for one type of smooth muscle, namely the anterior bryozoan retractor of the mollusc *Mytilus edulis* (ABRM) (Lowy and Hanson 1962, Schliebsch 1973).

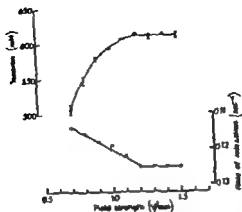
We report here experiments with the ABRM where we have established optimal stimulation procedures and eliminated spontaneous responses. Under such conditions the tension-length curve shows a feature hitherto seen only in frog skeletal muscle single fibre prepara-

mine oxidase. If so the activity of the dehydrogenase might be reduced during labor. It should be noted that diamine oxidase activity in human pregnancy reaches a plateau at the 7th month of pregnancy with high values up to term (Ahlmark 1944). It has been found in rats that the synthesis of putrescine is high in the placenta during the last trimester of gestation (Kim and Harris 1975, Guha and Jänne 1976). Much remains to be clarified regarding the role of diamine oxidase activity in the placenta not to mention other tissues containing this enzyme.

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1. Relation between stimulus strength and phasic contraction. The upper curve shows the dependence of phasic tension on a field strength for muscle maintained in 5 HT-containing ACS near rest at 0.5 V/cm. After mechanical and electrical equilibration the field strength was varied between 0.5 and 1.5 V/cm and 5 constant responses were recorded at each particular setting. Vertical bars ± 1 SD. In the lower curve the relation of maximum relaxation rate to field strength for the same muscle is shown. The points here represent the mean of 2 measurements (none deviation is less than 2%); this relaxation rate and active phasic tension becomes constant at about the same field strength about 1.2 V/cm.

Results

Determination of optimal stimulus strength for isometric phasic contractions

Lowy and Millman (1963) showed that stretching an unstimulated ABRM produces 2 kinds of tension: apparent resting tension (ART) which is abolished by application of 5 HT and resting tension (RT) which is considered to be the tension present in a 5 HT-treated muscle.

To produce phasic contractions, alternating current can be applied either to the untreated muscle or to a muscle maintained in a 5 HT-containing medium. The most important difference between these two preparations are that in the latter no ART is present and relaxation is appreciably faster (Lowy and Millman 1963).

To produce a smooth 'tetanic' response the muscle was stimulated at 10°C through transverse electrodes at L_9 with alternating current and a field strength of 1.5 V/cm. The stimulus period was 7 to 9 s. Intervals of 240 s were allowed between each period of stimulation. The manner of stimulation was continued and eventually completely uniform responses were obtained. Stimulation lasting longer than 7 to 9 s produced higher peak tensions (see section 3), but the muscle fatigued; we therefore did not stimulate for longer than 7 to 9 s. Once uniform responses were obtained the field strength was varied at every muscle length investigated and about 5 constant responses were recorded at each setting. Under these conditions a tension plateau was produced (Fig. 1 upper curve). The maximum tension developed per cross sectional area at L_9 (P_{90} in mN/mm²) depended on the size of the muscle, being higher the smaller the muscle. Conceivably smaller muscles have relatively less collagenous material. The maximum peak tensions measured varied from 600 to 1200 mN/mm². In the same responses the maximum relative rate of isometric relaxation (s^{-1}) was deter-

tions under special experimental conditions (Gordon *et al.* 1966), namely a plateau along which maximum tension is developed. We discuss various interpretations of ABRM plateau.

Methods

Very small specimens of *Mytilus edulis* were collected from local beaches and kept in an aerated sea water was circulated at 4°C. The ABRM muscles used were about 10 to 15 mm long and weighed between 3 and 10 mg.

Dissection

The animal was opened by cutting the adductor muscles. The foot and soft organs were removed. Intact the two ABRM muscles and all the posterior byssus retractor muscles (FBRM). A very light chain was tied to the latter just behind the byssus stem. One of the ABRM muscles was cut near its insertion into the byssal mass, and all the posterior retractor muscles were cut a few millimeters distal to gold chain. The shell to which the anterior end of the cut ABRM remained attached was removed. The other shell was clipped with bone forceps so as to leave only a small piece around the attached intact ABRM.

Measuring

The piece of shell was clamped into an adjustable perspex holder situated at the base of a perspex drum in which the muscle was suspended vertically by connecting the gold chain through a microsensor system to a strain gauge transducer. The overall compliance of this set-up was 1.0 $\mu\text{m}/\text{mN}$. In some cases we used a myograph with a stepping motor (Mullvany 1975) to change the muscle's length externally. The overall compliance of this set-up was 0.5 $\mu\text{m}/\text{mN}$. Two types of chambers were used depending on whether the muscle was stimulated by electrical shocks or by Acetylcholine (ACh). The ACh chamber is a smaller volume so that it could be filled and emptied within 1 to 3 s.

Oxygenated sea water at 10°C was circulated through the chamber at a rate of about 5 ml/min. A thermistor placed near the muscle the temperature was recorded throughout the experiment. Before start of each experiment the muscle was mechanically equilibrated by allowing it to hang with a load of 10 mN for 1 h. The osmolarity of the sea water was about 750 mOsm and its pH about 8. These values do not change significantly in the course of an experiment.

Stimulation

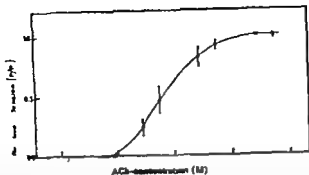
Depending on the method of stimulation the ABRM can produce kinds of isometric responses. Brief electrical shocks cause phasic contraction (Fletcher 1937) which is characterised by a fairly high relaxation rate. ACh induces a tonic 'catch' contraction (Twarog 1954) whose relaxation rate can be 2 orders of magnitude lower than in a phasic response (Lowy and Millman 1963).

To stimulate the muscle electrically alternating current was applied through transverse silver-silver chloride electrodes. The field strengths varied from 0.5 to 1 V/mm, depending on the size of the muscle. We also used condenser shocks with a multi-electrode assembly (Jewell 1959), and square wave pulses between 10 end electrodes (Lowy and Millman 1963).

Twarog (1954) described how the ABRM can be stimulated with ACh and relaxed with 5-hydroxytryptamine (5-HT). For experiments where we have used these drugs they were both freshly dissolved in sea water.

Muscle length changes

The muscle's length was measured in the chamber using a microscope with a calibrated eyepiece. We took as reference length (L_0) that length at which small resting tension (approximately 1 mN) can be detected in the presence of 5-HT at a concentration of $2.5 \cdot 10^{-6}$ M. This concentration is the lowest that completely abolished apparent resting tension (Lowy and Millman 1963) throughout the experiment. We know that if muscles were left in sea water medium containing higher 5-HT concentration they tended to deteriorate within a few hours. L_0 was established before the start of each experiment.



1. Relation between active tension and ACh concentration. The dose-response curve was obtained experimentally with 5 different muscles stimulated with ACh at L_0 . The ACh concentration is given in a logarithmic scale. The tension is expressed relative to maximum active tension, the mean of which is 970 mN/mm^2 . The vertical bars show $\pm 1 \text{ S.D.}$

dy and always to the original level. Using a concentration of $5.5 \cdot 10^{-4} \text{ M}$ ACh the die was stimulated at 20 min intervals until about 5 successive uniform responses were used. Muscles were rejected unless they could be equilibrated in this manner.

Using such equilibrated muscles we determined the dose-response curve for ACh by applying various concentrations of ACh and found that a tension plateau was produced at ACh concentrations higher than about $3 \cdot 10^{-4} \text{ M}$ (Fig. 3). However we also found that concentrations of ACh above $5.5 \cdot 10^{-4} \text{ M}$ caused irreversible damage. Therefore in our subsequent expts. on the muscle's active tension-length behaviour we only used ACh in a concentration of $5.5 \cdot 10^{-4} \text{ M}$, which produced more than 90% of the maximum tension.

Effect of length changes on the active tension production

Having established optimal activation conditions for both phasic and tonic responses we studied the muscle tension-length behaviour in both types of contractions. It is known from previous work that greater tensions are generated with ACh than with repetitive electrical shocks (Jewell 1959). This suggests that ACh produces more complete activation and we therefore describe here mainly our expts. on the ABRM's length-tension behaviour for tonic contractions.

Using ACh stimulation we investigated if the active tension-length behaviour of the ABRM was reversible over the muscle's normal working range. The latter we found to be between 0.8 and $1.2 L_0$ from expts. where the ABRM and PBRM were stimulated *in situ* (Fig. 4).

Fig. 5 illustrates the existence of active tension loss and plasticity—two phenomena that have been demonstrated previously in the ABRM (Abbott and Lowy 1958), as well as in mammalian smooth muscles (Dobrin 1973; Petersen and Paul 1974), and possibly also in obliquely-strained muscle (Miller 1975). The ABRM was stimulated with ACh once at each particular length. Both active and resting tension were determined. Starting at about $0.9 L_0$, the muscle was lengthened, shortened and lengthened again as indicated by curves 1 and 3 in Fig. 5. All length changes were effected in steps of $0.05 L_0$. It may be noted that as

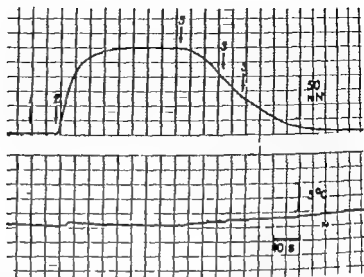


Fig. 2. Isometric tonic response of an equilibrated ABRM. Recorder trace of an ACh response at 10°C . The upper trace shows the active tonic tension developed at L_0 and the lower trace shows temperature variations. The calibrations are: tension 5.5 mN/div and temperature 0.5 $^{\circ}\text{C}/\text{div}$. Arrows indicate: (1) emptying of chamber (2) application of ACh, (3) emptying of chamber and washing with 5-HT-containing sea water.

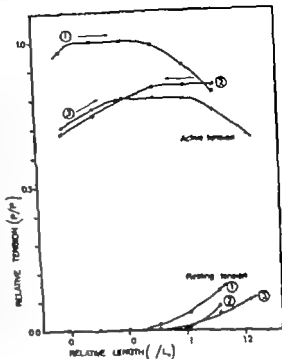
mined at each length as the slope of the relaxation phase of the tension-time curve at point of inflexion divided by the peak tension during the response concerned. We found the maximum isometric relaxation rate increased as field strength and tension was increased until a plateau was reached (Fig. 1 lower curve).

From these observations we conclude that for isometric phasic contractions at any particular muscle length the ABRM was stimulated optimally only when peak tension and relaxation rate reach plateau values. Using such conditions, completely uniform responses can be produced once every 240 s for periods up to 12 h. In previous work with the ABRM it proved impossible to obtain optimal activation (*i.e.* a plateau on the stimulus-response curve) for phasic responses using longitudinal field stimulation (Jewell 1959; Lowy & Millman 1963). With such stimulation we were also unable to obtain optimal stimulation. This is in line with results from experiments with frog skeletal muscle which have demonstrated that longitudinal field stimulation does not produce optimal stimulation (Stern-Kuch 1960).

2. Determination of optimal ACh concentration for isometric tonic contractions

After the usual mechanical equilibration and determination of L_0 the muscle was maintained in a 5-HT-containing sea water medium (5-HT concentration $2.5 \cdot 10^{-4}$ M) between applications of ACh. We found that in the absence of 5-HT it was difficult to obtain several successive uniform tonic responses after application of ACh. It takes a long time before the original resting tension level is re-established, and muscles also tend to fatigue. We therefore adopted the procedure illustrated in Fig. 2.

Before each stimulation the 5-HT-sea water medium was replaced by a sea water ACh-containing solution; the resulting tonic response levels out at a plateau. As soon as the plateau was reached ACh was washed out and replaced by the 5-HT medium; tension b



Plasticity and tension loss in the muscle associated with ACh. The muscle was equilibrated at about $0.9 L_0$ using Tonic responses were obtained at rest lengths by stretching the muscle passively in steps of $0.05 L_0$ up to $1.15 L_0$ and stimulating with ACh (1, square). The ABRM is then stretched passively in steps of $0.05 L_0$ and active tensions developed by ACh levels are recorded (curve 2). Finally the first procedure was repeated and produced curve 3 (triangles) corresponding resting tension curves are also shown. For further details see the active tension length curves (the shift along the length axis) and the downwards shift the tension axis (tension loss).

To test if activation is also involved in the phenomenon of tension loss we performed a series of experiments with ACh stimulation as shown in Fig. 6 a, b, c. The muscles were equilibrated at a certain length (reference length) and 3 to 5 constant responses were recorded (Fig. 6). After 10 min the muscle was passively stretched by $0.05 L_0$ and after 10 min it was again passively released to the reference length and 3 to 5 constant responses were recorded (○ in Fig. 6). The same procedure was then repeated, but this time the muscle was stimulated at the longer length (■ in Fig. 6) before being released to the reference length (stimulated again (□ in Fig. 6)).

Above and near L_0 it is evident that tension loss occurs when the muscle has been stimulated at a longer length (● and □ in Fig. 6 b, c). This tension loss is probably not due to some slip as no tension loss occurs if the muscle is only passively stretched but not stimulated at the longer length (○ and ○ in Fig. 6 b, c).

Above L_0 no such tension loss upon stimulation at the longer length takes place (Fig. 6 a). Whether this tension loss above L_0 upon stimulation at longer length is due to some kind of permanent rearrangement after which the system only "recovers" very slowly is currently under investigation.

These results demonstrate that for this particular kind of muscle it is impossible to obtain permanent and reproducible tension-length curves over the natural working range for different muscles unless a definite sequence in the length changes are performed.

Conditions in which a plateau in the active tension-length curve is obtained

In view of the above results we used one of the following two procedures to obtain the active

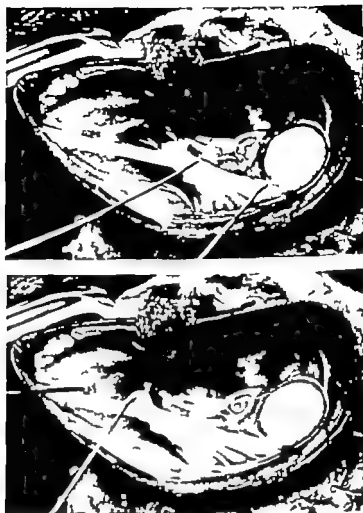
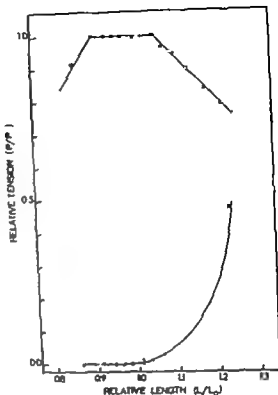


Fig. 4. The ABRM—working range. The pictures show a *Mollusca* whose soft organs have been removed, leaving intact the ABRM (to the left) and its antagonists, the PBRMs (to the right). The foot and siphon organ can also be seen. The upper picture shows a situation where the PBRMs are being stimulated actively with a.c. through two silver electrodes. After stimulation of the PBRM the ABRM was stimulated (lower picture). The length of the shell is about 30 mm.

resting tension was measured in the presence of 5-HT there is here no possibility of a contribution due to ART.

The phenomena of active tension loss and plasticity are most convincingly demonstrated by curves 1 and 2. Thus the active tensions produced at decreasing muscle lengths (active tension curve 2) all are below those previously given at the same length during the first part of the experiment, when the muscle length was increased (active tension curve 1). It is also evident that in the second part of the experiment the position of the plateau has shifted to the right along the length axis. The opposite tendency is apparent when the muscle is lengthened again by active and resting tension curves 3) but this time the active tension loss is relatively small.

The observed lengthening of the muscle is very likely due to plastic slip between the collagenous elements. This explanation is in line with the observation that the phenomenon is reversible.



Active and resting tension-length for some contractions. Results of muscles represented by 3 different symbols (○, ●, ○). The muscles were stimulated at 10°C by ACh at concentration of 5.5×10^{-4} M. Length changes of about $0.05 L_0$ were effected by a microtiter device. Inlet the muscle completely relaxed. Once ACh release was obtained at each length. The resting tension was measured in the rest of S-HT as described in the text. Points indicated by the symbols (○) and (●) were obtained from muscles relaxed from pre-stretched state. Points indicated by the symbol (○) were from muscle started from pre-relaxed state. The overall compliance set-up (including connections to stimulator) was 1 $\mu\text{m}/\text{mN}$. The plateau is at $0.15 L_0$.

As ascending limb, were active tension production increases up to an abrupt change at start of the plateau; (ii) a descending limb, whose onset is also abrupt. Table I shows that the plateau characteristics (i) remain essentially the same in phasic contractions and (ii) do not depend on whether the muscles used were in a pre-stretched or pre-shortened state. As regards (i), there is evidence indicating that the mechanism of calcium release is different in the two types of contraction (Sugi and Yamaguchi 1976). Moreover we have already mentioned the possibility that the muscle may be more easily activated by ACh than by repetitive electrical shocks. Nevertheless, Table I shows the existence of a plateau under both kinds of activation procedures. Under experimental conditions rather similar to ours the muscle's series elasticity has been found to be between 0.03 to $0.05 L_0/P$ (Lowy and Millman 1963 Jewell 1959). We therefore assume that in our expts. the isometrically stimulated muscle could shorten by at least $0.05 L_0$. To test the possible effects of such internal shortening on the existence of the plateau we performed an expt. where the muscle was allowed an extra shortening of $0.023 L_0$ during stimulation (quick release). Fig. 9 shows that this procedure left the plateau essentially unchanged. The downward shift of curve 3 relative to curve 2 suggests that deactivation of the observed recovered tension loss resembles that seen in similar expts. with frog skeletal muscle (Edman 1975). Finally we found that the plateau was still present under conditions when tension loss and plastic slip occurred (Fig. 5).

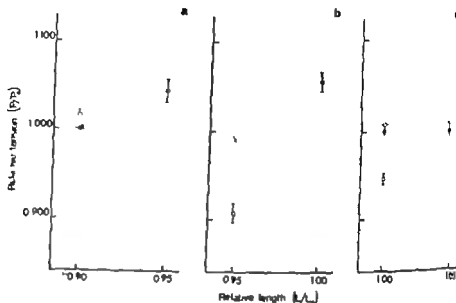


Fig. 6. Reversibility experiments. Experiments to illustrate how the tonic tension produced at a reference length is affected when the muscle has been passively stretched and then at the length either stimulated or not stimulated. The reference tension (●) is compared with the tension produced after a passive stretch and subsequent release of $0.05 L_0$ (○), and the tension produced after stretching to a longer length (□). Results from experiments performed at 3 different reference lengths are shown. Results from 5 muscles equilibrated at a reference length of $0.90 L_0$ (a). Results from 6 muscles equilibrated at a reference length of $0.95 L_0$ (b). Results from 3 muscles equilibrated at a reference length of $1.00 L_0$ (c). The points are the mean ± 1 S.E.

tension-length curve over the ABRM's natural working range (i) the muscle was started in a pre-shortened state and its length was passively increased (ii) the muscle was started in a pre-stretched state and its length was passively decreased.

Fig. 7 shows the active tension-length relation obtained for 3 muscles using ACh stimulation. The muscle's length was passively changed 10 min after ACh was washed out, and another 10 min. ACh was applied again at the new length. Starting either below or above the plateau the muscles were lengthened or shortened in steps of $0.02-0.05 L_0$.

The descending limb of the tension-length curve above the plateau was studied in detail in a series of expts. with muscles started from an extremely pre-stretched state (Fig. 8). As the muscles were all started from a pre-stretched state the points on the descending limb of the curve are unlikely to be affected by damage to the muscle which could conceivably occur in expts. with muscles started from a pre-shortened state. Linear regression analysis of the points on the descending limb of the tension-length curve (from 1.05 to $1.4 L_0$) gave a straight line with slope -1.11 ± 0.04 (mean \pm S.D.) and a correlation coefficient of 0.986 . The intercept on the length axis of this regression line is $1.93 L_0 \pm 0.03 L_0$ (i.e. the length at which no active tension would be developed).

We have determined the active tension-length curve for untreated and 5-HT-treated muscles and obtained the same results in both cases. The curves have the same general shape as that for tonic contractions (see Table 1).

Whichever of the procedures was adopted, all the active tension-length curves we obtained were similar (Fig. 7 and 8). (i) a small active tension is developed at the reference length.

1 Characteristics of the plateau region in active tension-length curves (plastic and hysteric).

of action	Starting condition (1)	Number of experiments	Plateau range Lower limit (2)	Upper limit
	pre-shortened	6	0.90 ± 0.06	1.06 ± 0.06
	pre-stretched	6	0.85 ± 0.03	1.06 ± 0.03
	pre-shortened	4	0.9 ± 0.01	1.04 ± 0.01
	pre-stretched	7	0.91 ± 0.04	1.04 ± 0.04

where the pre- or post-tension of L_0 and are the mean \pm S.D. (1) Experiments started either at about (pre-shortened) or 1.2 L_0 (pre-stretched). (2) Plateau range is the range of muscle lengths over which to 1.00 P is developed. (P is the maximum tension at L_0 in mN/mm².) The muscle lengths at which action begins (upper limit) and ends (lower limit) are indicated.

that the whole muscle behaves functionally as a single cell, that the length of the thick thin filaments is constant, and that at any particular muscle length the sarcomeres in cell have the same amount of filament overlap. The idea that under suitable activation the ABRM could respond as a single cell is supported by recent work which is that, as in vertebrate smooth muscle, the cells in the ABRM are electrically connected by gap junctions (Twiss, Dewey and Hidaka 1973). Electron microscopy (Sobieszek 1973) indicates that the filaments in the ABRM are organised in an arrangement comparable to sarcomere, and the available evidence does not exclude the idea that both the thick and the thin filaments have uniform lengths (Sobieszek 1973). In fact, in vertebrate smooth muscle it has been established that the length of the thick filament is constant (Sobieszek and Somojai 1975). The presence of sharp corners on the ABRM tension-length curve makes it unlikely that the plateau can be explained as the sum of individual tension-length curves from cells whose lengths are statistically distributed.

We here show that the ABRM plateau can be explained on a sliding filament mechanism exactly similar to that for striated muscle. That such a mechanism operates in the ABRM is strongly suggested by structural evidence both from electron microscopy (Lowy and Hanson 1972) and X-ray diffraction studies (Lowy and Vibert 1972). Electron microscopy has also shown (Szent-Gyorgyi *et al.* 1971) that there is reversal of polarity in the middle of the thick filament, and that the thin filaments have opposite polarity on either side of a structure called a dense body (which is equivalent to a Z line (Lowy and Hanson 1962)).

By analogy with the sliding model for frog skeletal muscle the simplest explanation for the ABRM tension plateau would be in terms of a cross-bridge free region of sufficient length in the middle of the thick filament (Gordon *et al.* 1966). Estimates for the lengths of the thick filaments in the ABRM vary between 10 and 30 μ m (Sobieszek 1973, Lowy and Hanson 1972), and the ABRM plateau would therefore require a region free of cross-bridges of 5–4.5 μ m. Such regions have not yet been found and we consider this possibility to be unlikely on structural grounds.

In mechanically skinned cardiac fibres Robinson and Winegard (1977) found that the lengths of thin filaments vary by as much as 0.6 μ m within single sarcomeres in a single

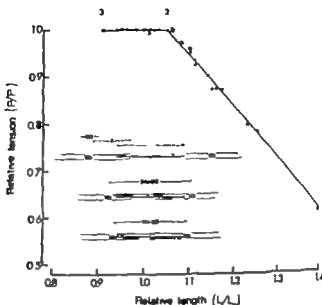


Fig. 8. Relation between active tension and muscle length for tonic responses. The points are from experiments with 6 different muscles stimulated by ACh at a concentration of $5.5 \cdot 10^{-4}$ M. Step in steps of $0.05 L_0$ was effected by a micrometer device. One ACh response was obtained at each. The overall compliance of the set-up (including connections to the muscle) was $1 \mu\text{m}/\text{mN}$. Only points obtained from experiments started with muscles in a pre-stretched state are shown. Also shown is the length-tension curve (full line) predicted from the model illustrated in inset. The straight line of the descending limb of the predicted length-tension curve is -1.20 ± 0.06 S.D. (The slope here is due to the experimentally found variations in plateau range, see Table 1.) Linear regression of the points obtained over that region of muscle lengths (from $L/L_0 = 1.05$ to 1.40) gives a slope with slope -1.11 ± 0.04 S.D. (The length at which no active tension would be developed as calculated the experimental points is $1.93 L_0 \pm 0.03 L_0$). The numbers and arrows along the top refer to the diagrams in the inset.

Inset. Siding filament model to explain the ABRM plateau. The drawings show schematically the arrangement in sarcomere at critical stages in the muscle's length-tension behaviour: (1) Dense sarcomere. A length of thin filament on one side of dense body \approx length of thick filament, length of the dense body (2) Arrangement of filaments in a sarcomere at the beginning of the plateau (equivalent to $1.05 L_0$ in Fig. 8 and upper limit in Table 1). Here the thin filaments just meet at the ends of the sarcomere. The length of the sarcomere is $A + DB$. (3) Arrangement of filaments at the end of plateau (equivalent to $0.91 L_0$ in Fig. 8 and lower limit in Table 1). The ends of the thick filament are at the dense bodies. Sarcomere length is $M + DB$.

Discussion

The finding of a plateau with sharp corners on the active tension length curve of a smooth muscle is remarkable and suggests a highly ordered filament arrangement in ABRM muscle cells. The relative length at which this plateau begins and ends is most the same for tonic and phasic contractions and for muscles started in a pre-shortened or pre-stretched state (Table 1). A plateau is also present when the muscle is deactivated by release (Fig. 9) and under conditions where tension loss and plasticity occur (Fig. 9). The presence of a plateau under these various conditions leads us to attempt an explanation in terms of events at the filament level. To do this we require a mechanism by which, in the plateau range, changes in muscle length are not accompanied by changes in the number of cross-bridges formed between the thin and thick filaments.

terminates when the dense bodies reach the ends of the thick filaments (but not necessarily them). Here the ends of the thin filaments begin to overlap thick filaments of the 'wrong' polarity right from the beginning of the plateau.

In the frog model we assume that cross-bridges cannot form with thin filaments that overlap thick filaments of the 'wrong' polarity. In the frog model the abrupt loss of tension which occurs right after the end of the plateau is believed to be due to interference with cross-bridge formation when double overlap begins because of strong mechanical constraints in the highly regular filament lattice of the frog muscle (Gordon *et al.* 1966, Huxley 1965). In the ABRM there are fewer such constraints, there is no regular lateral packing of the filaments (Sobieszek 1973, Lowy and Hanson 1962) and for each thick filament there are about 17 thin ones (Sobieszek 1973) and some of these do not surround thick filaments, but lie in small lattices between them, as in vertebrate smooth muscle (Ashton *et al.* 1975). We believe that in both these types of smooth muscle spatial considerations will not lead to interference with cross-bridge formation at double overlap.

Using the data from ACh contractions, then according to our explanation the upper limit of the plateau (see Table I) is equivalent to a sarcomere length of $2A + DB$ (where A is the length of the thin filaments on one side of the dense body whose length is denoted DB) $2A + DB = 1.05 L_0$. The lower limit of the plateau (see Table I) is equivalent to a sarcomere length of $M + DB$ (where M is the length of the thick filament) $M + DB = 0.91 L_0$. From electron microscope data (Sobieszek 1973) we take: (3) $2A/DB = 12.3$. From (1), (2), we then obtain the non-overlap length, $2A + M + DB = 1.88 L_0$ and the ratio of thin to thick filament length, $A/M = 0.38$.

Assuming that the cross-bridges are evenly distributed along the thick filament, the ascending part of the active tension-length curve for the frog model will be a straight line (Gordon *et al.* 1966). Making the same assumption for the ABRM the slope of this line can be derived from the calculations above which give $1.88 L_0$ as the length of the stretched muscle where there would be no overlap. Fig. 8 shows that the experimentally determined points lie reasonably close to the calculated curve. Unfortunately the figure of $1.88 L_0$ cannot be checked by expts. with whole muscle because the resting tension rises very steeply above $1.2 L_0$ (Fig. 2) and the muscle breaks when stretched to lengths greater than about $1.5 L_0$.

We are aware that there are other plausible explanations of the ABRM's tension-length behaviour including the plateau. Thus in the absence of constraints such as an M-line or a Z-disk, the filaments might undergo some kind of rearrangement relative to one another as the muscle is stretched. 'Shearing' (Rosenbluth 1972) or a mechanism that enables thick filaments to change their thin filament partners (Poulsen and Lowy 1975) could account for the ABRM plateau. But such mechanisms require much more elaborate assumptions than the proposed model.

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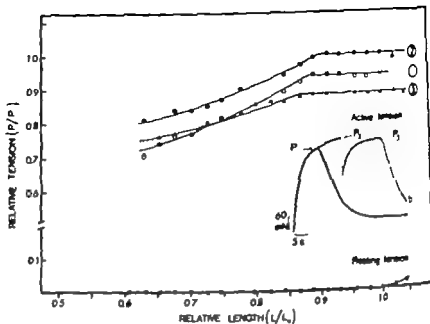


Fig. 9 Active and resting tension-length curves obtained from a quick-release experiment. The muscle was pre-stretched to $1.05 L_0$ and stimulated by alternating current for 9 s every 240 s until it produced responses. It was then stimulated for 35 s and released after 20 s by $0.023 L_0$ at a speed of 50 mm/s. This procedure was repeated until the muscle had shortened to about $0.6 L_0$. The myograph and same as that described by Mulvany (1975). The compliance of this set-up (including connecting muscle) was $0.5 \mu\text{m/mN}$. All tensions are taken as relative to the maximum peak tension obtained response at L_0 (P_0 in insert). Curve 1 represents the mean of 2 uniform contractions in a 9 s rest in insert). Curves 2 and 3 were obtained using, respectively the 20 s and 35 s response (P and P_0 in insert). Insert: Oscilloscope record from a quick-release experiment. Trace a: Normal phasic response stimulus. Trace b: Response to a 35 s stimulus after 20 s the muscle was released by $0.023 L_0$. Normal phasic response at the shorter length which is identical to Trace a.

myofibril and this led them to conclude that the active tension produced in their prep depends not only on overlap but also on the distribution of lengths of the thin filaments. Active tension-length curves derived on the basis of these considerations show a plateau about three times as wide as that obtained in isolated whole skeletal muscle fibres. The corners between the plateau and the ascending and descending limbs of their tension-length curves are very rounded. The presence of thin filaments having a length is therefore not very likely to account for the ABRM plateau we have observed.

The ABRM plateau can, however, be simply explained by a sliding filament mechanism resembling that in frog skeletal muscle except that double overlap of the thin filaments with the ABRM does not interfere with cross-bridge formation between thick and thin filaments of the appropriate polarity. In what follows this explanation will be discussed with reference to the sliding model for frog skeletal muscle (frog model) (Huxley and Niedergerger 1954; Gordon *et al.* 1966). In the frog model the plateau begins respectively when the thin filaments reach the beginning and the end of the cross-bridge region. Further shortening brings the ends of the thin filaments to overlap thick filaments of the 'wrong' polarity ('double overlap'). According to our explanation (Fig. 8) the ABRM plateau begins when the thin filaments reach the middle of the thick filament

Prostaglandin content in blood and lung tissue during alveolar hypoxia

By

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Abstract

BERG, E., VAAGE, J., BJERTNÆS, L., HAUGE, A. and GAUTVIK, K. M. Prostaglandin content in blood and lung tissue during alveolar hypoxia. *Acta physiol. scand.* 1978, 102, 181-190.

The aim of the present work was to investigate whether prostaglandins (PGs) are synthesized and released in isolated blood-perfused rat and cat lungs secondary to vasoconstriction induced by alveolar hypoxia. Lungs are perfused with autologous blood with constant volume inflow via the pulmonary artery in a recirculating system. They were ventilated with constant volume positive pressure, and acute alveolar hypoxia was induced by ventilation with gas containing 3% O_2 . A superfusion bioassay technique was used to measure PG-like activity in the perfusate from the lungs, the blood being re-oxygenated before being used for the assay. The oxygenator prevented the perfusate hypoxia induced by ventilation hypoxia affecting the assay tissues. The assay tissues were rat stomach strip, rat colon and chick rectum. They are sensitive to calibrating doses of 0.5-1 $\mu\text{g/ml}$ PGF_2 and 1-2 $\mu\text{g/ml}$ $PGF_{1\alpha}$. In another series of experiments PGs of the F-series were measured in lung tissue from normoxic and hypoxic lungs with radioimmunoassay techniques. No increase in PG-like activity could be detected in the venous effluent by means of bioassay during hypoxia, nor was the lung tissue content of immunoreactive PGF increased by hypoxia. General findings indicate that alveolar hypoxia does not stimulate PG-synthesis in lungs, refuting that it is an important mediator of the pulmonary vasoconstrictor response to alveolar hypoxia. It is concluded that PGs play no significant role in producing thepressor response to alveolar hypoxia.

The mechanism whereby acute alveolar hypoxia elicits pulmonary vasoconstriction still remains to be properly clarified. Conflicting observations have been reported concerning a role for prostaglandins (PGs). These substances are possible mediator candidates for thepressor response to hypoxia since the lungs have a large capacity for synthesis and release of PGs secondary to various mechanical and chemical stimuli (Piper and Vane 1971, Piper 1974). Moreover $PGF_{1\alpha}$ as well as various PG-endoperoxide analogs are potent constrictors of the pulmonary circulation (Ånggård and Bergström 1963, Rowe *et al.* 1976). Furthermore, intrapulmonary synthesis of PGs induced by infusion of arachidonic acid also causes vasoconstriction in the pulmonary circulation (Wicks *et al.* 1976). Said and coworkers (1974) reported release of PG-like substances from isolated, perfused cat lungs during ventilation with a hypoxic gas mixture. They also found that aspirin, which inhibits PG-synthesis (Vane 1973), reduced the vasoconstriction induced by hypoxia in

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anesthetized cats, and they suggested that vasoconstrictor PGs mediate this response. Vaage and Hauge (1975) argued against this hypothesis and reported experiments on rats (Vaage, Bjertnæs and Hauge 1975) which showed that 3 different inhibitors of PG synthesis had no effect on the response to acute hypoxia in lungs from this animal. In a reply Saito and Hara (1975) reported additional experiments which again gave as a result that aspirin (not indomethacin) reduced the pressor response to acute alveolar hypoxia. They suggest that the conflicting results reported by Vaage, Bjertnæs and Hauge (1975) could be attributable to species differences.

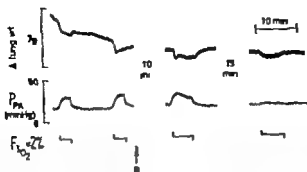
Anderson *et al.* (1972) and Even *et al.* (1975) reported that no increase in PG content was found in blood neither from calves nor from humans during hypoxia-induced pulmonary hypertension. Furthermore, we and others have previously demonstrated that PG synthesis is inhibited in different types of lung preparations from several species (Bjertnæs and Hauge 1975, Tyler *et al.* 1975, Weir *et al.* 1976a, Hales *et al.* 1977, Even *et al.* 1975). It has therefore been suggested that the response to hypoxia is modulated by pulmonary synthesis and release of vasodilatory PGs (Vaage *et al.* 1975). Thus, the evidence so far suggest that PGs does not mediate the vasoconstrictor response, but rather act to reduce it, preventing the pulmonary arterial pressure to become excessively high.

In the present work we have aimed at determining once and for all the possible role for vasoconstrictor and vasodilator PGs in the pressor response to alveolar hypoxia. We have included cat lungs in addition to rat lungs and we have utilized a bioassay technique similar to that of Said *et al.* (1974). In addition we have determined lung tissue content of PGF with radioimmunoassay. Preliminary reports of this study have previously been presented (Bjertnæs *et al.* 1976, Vaage *et al.* 1976).

Methods

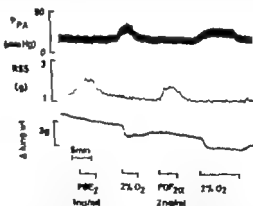
Rat lung preparation. 1 breed albino rats (local strain), weighing between 180–220 g. were used. The donor animal was anesthetized with pentobarbitone sodium i.p. (3–4 mg/100 g b.w.t.), a tracheotomy performed, and the chest opened during positive pressure ventilation. The trachea, the lungs and the larger intrathoracic vessels were dissected free and heparin was injected (100 I.U. in 0.5 ml saline) into the left ventricle before ligation of the caval veins. Stainless steel cannulas were placed in the pulmonary artery and the left atrium. The cannulas were fastened by a tight ligature around the heart, and the rats were transferred to a constant temperature (37°C) perspex chamber. The lungs were freely aerated by a string fastened to the heart ligature and perfused in a recirculating system with 40–50 ml heparinized (10 I.U./ml) blood, obtained by cardiac puncture of ether-anesthetized donor rats. All the four perfused lungs which were used with bioassay were perfused with a volume flow of 10 ml/min in the pulmonary artery by means of a roller pump. The 5 lungs used for radioimmunoassay had a flow of 15–20 ml/min. Perfusion was begun within 12 min after the interruption of the animal's own circulation. Pulmonary pressure (P_{PA}) was measured by Statham P-3Db pressure transducer connected to a Grass polygraph (Model 7B). A schematic drawing of the experimental set-up is shown in Fig. 1.

Cat lung preparation. Cats weighing 1.9–2.1 kg were used as lung donors. They were anesthetized with pentobarbitone sodium i.p. (30–40 mg/kg b.w.t.). The lungs were dissected free as described for rats. As blood donors were used cats (b.w.t. 3–4.5 kg) for each perfusion experiment. Also the blood donors were anesthetized with pentobarbitone. They were heparinized with 300 I.U./kg b.w.t. and exsanguinated by cardiac puncture. To each 100 ml of blood was then added 1 000 I.U. heparin dry powder (LEO). Approximately 200 ml of blood was used in the perfusion circuit. The cat lungs were perfused



The figure demonstrates the effect on the pressor response to hypoxia in rat lungs of including the resistor and bypass circuit in the perfusion system. This particular preparation, as studied for physiological reasons and is not included in Table I. From the top are illustrated changes in preparation weight (Δ lung wt) and pulmonary arterial pressure (P_{PA}). It began with the preparation weight was 1 (a regular observation). Two 3 min periods of alveolar hypoxia ($P_{A_{O_2}} = 2\%$) elicited good pressor responses, but only the perfusion circuit was open. At point B the oxygenator-bypass circuit was introduced. The 3 min later 3 min period of hypoxia still caused a pressor response, but the peak pressure was maintained. After 36 min the pressor response to hypoxia had almost vanished. The right deflection, which is changes in pulmonary blood volume, caused by alveolar hypoxia, is better maintained and will be an independent type of response.

A pressor response to alveolar hypoxia in isolated rat lungs follows a characteristic pattern (Hinge 1968 a, b). At the beginning of perfusion standardized "hypoxic tests" elicit no or very small pressor responses. After a few tests the response gradually increases until there is a maximum effect for the given time period of hypoxia. Several equal responses can then be obtained before a gradual decline in responsiveness occurs.



1. Effects of ventilation hypoxia on isolated rat lungs perfused with blood at constant volume inflow. At the top are demonstrated recordings of pulmonary arterial pressure (P_{PA}), tension of the most sensitive rat stomach strip (RSS), which was superfused by the venous effluent, and change in preparation weight (Δ lung wt). First the bypass circuit is calibrated with 1 mg/ml of POE_2 infused into the superfusing blood. Then comes a 4 min period with alveolar hypoxia ($1 - O_2$), causing an increase in P_{PA} , this is the "chemical" pressor response to acute hypoxia. Hypoxia also induced rapid reversible reduction in preparation weight, but had no effect on the RSS. After this period of hypoxia a liberating dose of 2 mg/ml of $PGF_{2\alpha}$ was infused. Finally the effects of a 10 min period of ventilation hypoxia ($2\% O_2$) is demonstrated. Again there is marked pressor response and reversible reduction in preparation weight without detectable effects on the bypass tissue.

Oxygenator The blood superfusing the bioassay tissues (10 ml/min) was pumped through an oxygenator to the bioassay tissues (Fig. 1). With cat lungs 10 ml/min of the venous effluent as pumped to the oxygenator the rest of the perfusate flow was shortcircuited to the inflow reservoir. Using rat lungs the perfusate outflow (10 ml/min) was passed through the oxygenator and was used for bioassay.

The oxygenator was made in The Mechanical Workshop, The Preclinical Institute, Umeå, Sweden. It consisted of a glass tube with a sinuous internal surface rotating inside a water jacket (Fig. 2). The internal surface of the tube was siliconized with Siliclad (Clay Adams, N. J. USA). The axis of rotation was at an angle of 30 degrees with the horizontal plane. Thus blood delivered in the top of the tube, was spread over the internal surface of the tube and then running slowly downward to a thermostated reservoir. In this semiclosed reservoir the oxygenator gas was delivered through a tube, the gas containing 5% in O_2 . The gas flowed upwards inside the internal lumen of the sinuous tube at a rate of 5 l/min. The wall of the tube was covered with a blood film. Gas and blood came in contact during counterflow. The gas outlet was on top of the oxygenator, the blood outlet being at the bottom (Fig. 2).

PO_2 in the venous effluent decreased from 110–120 mmHg in the control situation to approximately 30 mmHg during ventilation with the hypoxic gas mixture. The oxygenator however maintained a constant PO_2 (500–525 mmHg) in the blood superfusing the assay tissues regardless of the alveolar PO_2 .

Bioassay A modification of the superfusion organ technique (Vane 1969) was used for the bioassay of PGs. Our bioassay arrangement has been described in detail by Vaage, Scott and Wiberg 1972.

When leaving the oxygenator the blood was pumped by one channel of the roller pump (D.D.) through a silastic tubing, with an injection point for infusion of standard doses of PGs. The tubing was heated with a water jacket to 37°C before reaching the assay tissues (Fig. 1). The assay tissues were rat stomach strip (RSS), rat colon (RC), and chick rectum (CR) (Fig. 1). Before blood superfusion the tissues were treated for 2 h in a Krebs-Ringer solution containing pharmacological blockers against the action of catecholamines (α - and β -receptors), histamine (H_1 -receptors), serotonin and acetylcholine (Vaage *et al.* 1978). The isometric tension of the assay tissues was measured with mechanical elements (Vaage *et al.* 1978). After passing over the tissues the blood was returned to the reservoir.

Calibrating doses of PGs were infused at the injection point. The assay tissues contracted to 0.5–1 g of PGE_2 and 1–2 ng/ml of $PGF_{1\alpha}$.

Radioimmunoassay 5 additional rat lungs were prepared as described and perfused with heparinized blood but without the circuit for bioassay and the oxygenator. 4 min tests of alveolar hypoxia with air of 5 min were performed throughout the perfusion period. When marked pressor response was seen 3 pairs of rat lung were rapidly removed from the perfusion circuit after 3½ min of alveolar hypoxia, frozen in liquid nitrogen cooled to about -140°C, 4–5 min passed between the stop of perfusion and the removal of the lungs to liquid nitrogen. Two other rat lungs were perfused for similar periods of time and served as controls. They served as controls and were processed exactly as the hypoxic lungs. Approximately half of the weight of the frozen preparation was used for tissue processing. Only the peripheral part of lung tissue was used, thus avoiding large blood vessels and large airways.

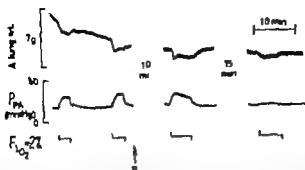
The lung tissue was homogenized with Dounce homogenizer (2 min, 0°C) in 5 ml 0.15 M NaCl and 0.1 M HCl (5:2) giving a weight concentration of tissue of 200 mg/ml. Extraction of PGs was performed with 1 ml of the homogenate (1 ml) that was transferred to tubes containing 2.6 ml ethyl acetate (1:1) and mixed on a wristmixer (10 min). To each tube was added 3 ml 0.15 M NaCl and 2 ml ethyl acetate and the tubes were centrifuged (50 000 g, 30 min, 4°C). A portion (1 ml) of the ethyl acetate phase was evaporated under nitrogen at 50°C, the residue dissolved in 2.5 ml 0.01 M Tris-HCl (pH 7.4) and about 50–150 µl taken for radioimmunoassay.

Before the labelled $PGF_{1\alpha}$ (New England Nuclear Corporation) was used for assay incubation, it was purified on silicic acid columns (0.5 g, 100 mesh). The antiserum did not distinguish between the two isomers but showed no cross reaction with PGA , PGB , PGE_1 , PGE_2 , nor with the dithyrol- or ketone-derivatives of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Gautvik *et al.* 1978).

All determinations were carried out in triplicates which differed less than 15%.

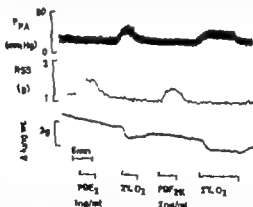
Results

Baseline values of pulmonary arterial pressure (P_{PA}) and flow as well as pressor response to alveolar hypoxia are given in Table I. In cat lung preparations acute weight reduction ranging from 0.4 to 1.3 g occurred in all the periods with hypoxia concomitant with increase in P_{PA} (Figs. 2 and 3).



The figure demonstrates the effect on the pressor response to hypoxia as cat lungs of lacking the inter and accessory circuit at the perfusion system. This particular preparation was studied for technical reasons and is not included in Table I. From the top are illustrated change in preparation (Δ lung wt) and pulmonary arterial pressure (P_{PA}). T. began with the preparation weight was (a regular observation). Two 3 min periods of alveolar hypoxia ($F_{IO_2} = 10\%$) elicited good pressor responses when only the perfusion circuit was open. At point B the oxygenator-bypass circuit was initiated. Ten min later 5 min period of hypoxia still caused a pressor response, but the peak pressure was attenuated. After 16 min the pressor response to hypoxia had almost vanished. The weight reduction, noted as changes in pulmonary blood volume, caused by alveolar hypoxia was better maintained and will be an independent type of response.

As pressor response to alveolar hypoxia in isolated rat lungs follows a characteristic pattern (Hauga 1968 a, b). At the beginning of perfusion standardized "hypoxic tests" induce no or very small pressor responses. After a few tests the response gradually increases until there is a maximum effect for the given time period of hypoxia. Several equal sized responses can then be obtained before a gradual decline in responsiveness occurs,



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PO_2 in the venous effluent decreased from 110–120 mmHg in the control situation to approximately 30 mmHg during ventilation with the hypoxic gas mixture. The oxygenator however maintained constant PO_2 (500–525 mmHg) in the blood superfusing the assay tissues regardless of the alveolar PO_2 .

Bioassay. A modification of the superfusion organ technique (Vane 1969) as used for the lungs PGs. Our bioassay arrangement has been described in detail by Vaage, Scott and Wiberg 1978.

When leaving the oxygenator the blood was pumped by one channel of the roller pump (8 ml) through a plastic tubing, with an injection point for infusion of standard doses of PGs. The tubing was heated with a water jacket to 37°C before reaching the assay tissues (Fig. 1). The assay tissues were rat stomach strip (RSS), rat colon (RC), and chick rectum (CR) (Fig. 1). Before blood superfusion the tissues were treated for 2 h in a Krebs-Ringer solution containing pharmacological blockers against the action of catecholamines (α and β -receptors), histamine (H-receptors), serotonin and acetylcholine (Vaage *et al.* 1978). The isometric tension of the assay tissues was monitored with sensitive elements (Vaage *et al.* 1978). After passing over the tissues the blood was returned to the reservoir of the oxygenator.

Calibrating doses of PGs were infused at the injection point. The assay tissues contracted to 0.5–1.0 g of PGI_2 and 1 ng/ml of $PGF_{2\alpha}$.

Radioimmunoassay. 5 additional rat lungs were prepared as described and perfused with heparinized blood but without the circuit for bioassay and the oxygenator. 4 min tests of alveolar hypoxia with arrest of 5 min were performed throughout the perfusion period. When a marked pressor response was present 3 pairs of rat lung were rapidly removed from the perfusion circuit after $1\frac{1}{2}$ min of alveolar hypoxia and frozen in liquid freon cooled to about -140°C. 4–5 passed between the stop of perfusion and the removal of the lungs to liquid freon. Two other rat lungs were perfused for similar periods of time and stored under normoxic conditions. They served as controls and were processed exactly as the hypoxic lungs. Approximately half of the weight of the frozen preparation was used for tissue processing. Only the peripheral part of lung tissue was used, thus avoiding large blood vessel and large airways.

The lung tissue was homogenized with a Dounce homogenizer (2 min, 0°C) in 5 ml 0.15 M NaCl and 0.1 M HCl (5:2) giving a weight concentration of tissue of 100 mg/ml. Extraction of PGs was performed with a fraction of the homogenate (1 ml) that was transferred to tubes containing 2.6 ml ethyl acetate:methanol (1:1) and mixed on a whirlmixer (10 min). To each tube was added 3 ml 0.15 M NaCl and 2 ml ethanol and the tubes were centrifuged (50 000 g 30 min, 4°C). A portion (3 ml) of the ethyl acetate phase was evaporated under nitrogen at 50°C, the residue dissolved in 2.5 ml 0.01 M Tris-HCl (pH 7.4) and aliquoted 50–150 μ l taken for radioimmunoassay.

Before the labelled $PGF_{2\alpha}$ (New England Nuclear Corporation) was used for assay incubation, it was purified on silicic acid columns (0.5 g, 100 mesh). The eluent did not distinguish between the F and G compounds but showed no cross reaction with PGA_2 , PGB_2 , PGE_2 , PGE_3 , nor with the dihydro- or ketone analogues of $PGF_{2\alpha}$ and $PGF_{2\beta}$ (Gautvik *et al.* In preparation).

All determinations were carried out in triplicates which differed less than 15%.

Results

Baseline values of pulmonary arterial pressure (P_{PA}) and flow as well as pressor response to alveolar hypoxia are given in Table I. In cat lung preparations acute weight reduction ranging from 0.4 to 1.3 g occurred in all the periods with hypoxia concomitant with increase in P_{PA} (Figs. 2 and 3).

ur hypoxia. During the recordings of pressor responses in cat lungs reversible reduction in weight occurred concomitant with the increase in pulmonary arterial pressure (2 and 3). To our knowledge this effect has not previously been published. Such weight losses most likely represent reversible reductions in blood volume (Hauge *et al.* 1967 a). Reduction of pulmonary blood volume has been observed in intact animals during hypoxia (Aarneth, Karlson and Be 1975, Karlson and Aarneth 1976 and Aarneth and Be 1977). From Fig. 1 it appears that when the pressor response has almost disappeared, there is still a significant weight reduction during hypoxia. This may indicate that a reduction in blood volume due to vasoconstriction of capacitance vessels occurs independent of the pressor response. Similar dissociation between resistance and capacitance vessels has previously been described (Hauge *et al.* 1967 b).

2 also illustrates the finding that when the bypass circuit is opened the pressure decrease caused by alveolar hypoxia rapidly declines, allowing only a few good pressor responses to be elicited with the bypass system in action. This observation should be put in context with that of Duke and Millick (1952). They perfused cat lungs for as long as 1 h with continuously increasing pressor responses to hypoxia. We have no definite explanation for this discrepancy. Changes in the blood as it comes into contact with the wet surfaces of the oxygenator and the assay tissues may play a role. Reeves and Grover (1964) have reported that small amounts of endotoxin block acute pulmonary hypertension induced by hypoxia in dogs. The assay tissues may certainly contain enough endotoxin of bacterial origin to block the hypoxic response.

The effect of endotoxin has been suggested to be mediated through synthesis of vasoconstrictor PGs (West *et al.* 1976 b). However we did not find any evidence of a progressive release of PGs during perfusion in our setup. Contact of the perfusate blood with foreign surfaces such as polyvinylchloride (PVC) will also prevent the response (Duke and Vane 1971).

We now turn to our main findings, namely those regarding pulmonary PG-synthesis during hypoxia. Perfused rat and cat lungs have considerable capacity to release PGs primarily in a variety of steroids (Bakke and Vane 1974). Whether intrapulmonary synthesis and release of PGs will cause a constrictor or a dilator response depends on the balance between the different subgroups of prostaglandins which are released. The release of PGs from the lungs is dependent on a rapidly induced biosynthesis, since only negligible amounts of PGs are stored in lung tissue (Piper and Vane 1971).

Our results show that in both rat and cat lungs perfused with blood, the vasoconstriction induced by ventilation hypoxia is not accompanied by detectable PG-synthesis. From studies on perfused cat lungs Said and coworkers (1974) on the other hand, claimed that biosynthesis of PGs during alveolar hypoxia "suggested the appearance in the perfusate of PGE-like compounds, with the activity of up to 50 ng of PGE₂ per milliliter" and the presence of F-like compounds, with the activity of up to 50 ng of PGF_{2α} per milliliter" during ventilation hypoxia. An important methodological objection is that these investigators did not use an oxygenator and thus exposed the assay tissues to the direct effects of hypoxia (Said *et al.* 1974, Fig. 1). Any effects caused by PGs in the perfusate may thereby be obscured, and even it is impossible to find out whether the PG levels are unchanged during hypoxia. Further

TABLE I Vasoconstrictor responses to alveolar hypoxia I Isolated lungs.

Expt no	Species	Blood flow (ml/min)	Baseline art. pressure (mmHg)	Peak rise in art. pressure during sequential hypoxic tests (mmHg)			
				I	II	III	IV
1	rat	10	20	9	9	11	7
	rat	10	38	6	4	3	1
3	rat	10	—	8	8	11	11
4	rat	10	1	9	10	9	8
5	cat	40	14	7	7	5	—
6	cat	15	15	13	10*	9*	—

Hypoxic period 10 min.

until finally alveolar hypoxia no longer produces a pressor effect. Cat lungs on the other hand can be perfused for several hours with increasing pressor responses (Dole and K 1952)

Both patterns are, however, affected when the oxygenator and the bioassay system are included in the perfusion circuit: the period in which hypoxia induces responses is shortened, so that only a small number of such responses can be obtained each pair of lungs. Also when the oxygenator and the bioassay circuit are included in the period with good pressor response, the responses rapidly decline. This was proved when rat lungs were used, most likely because the whole perfusate flow passed through oxygenator and bioassay circuit. With cat lungs only a small fraction (10 ml/min) of perfusate flow (152–240 ml/min) was led through the oxygenator and over the bioassay tissues. Nevertheless, also in this situation the number of pressor responses which could be elicited was drastically reduced. This was tested separately in 2 cat lungs. In both the pressor response to hypoxia rapidly declined after opening to the bioassay-on-perfusion circuit, as illustrated in Fig. 4.

During the total of 28 periods of hypoxia studied (Table I) there was never an cessation of any of the 3 assay tissues in spite of marked and fully reversible pressor responses. Even in the cases with 10 min periods of hypoxia the baseline tension of the assay tissues remained stable. Fig. 3 shows effects of ventilation hypoxia in cat lungs. The bioassay response is represented by the recording of RSS tension since this is the tissue most sensitive to PG.

Periods of ventilation hypoxia after the pressor responses had disappeared did not produce any PG-like activity.

Lung tissue content of PGF was measured with radioimmunoassay. The 2 control lungs frozen during ventilation with 5% CO₂ in air contained 114 and 81 ng PGF per g wet tissue, respectively. The 3 test lungs frozen during ventilation hypoxia and with no pressor response contained 62, 80 and 69 ng PGF per g wet lung tissue.

Discussion

In this work we have mainly focused on alveolar hypoxia and PG-synthesis. Some additional observations relate, however, to other aspects of the pulmonary vasoconstrictor res-

xh muscle, thus giving an increased pressor response to acute alveolar hypoxia (Haug and Hauge 1975, Tyler *et al.* 1975, Weir *et al.* 1976 a, Hales *et al.* 1977). This modulating action is rather small and has no relevance in elucidating the role of the vasoconstrictor response to alveolar hypoxia. Our results show that the pulmonary vasoconstrictor response to ventilation is mediated by stimulation of PG synthesis.

We are grateful to the generous gift of Professor Lawrence Levine, Harvard University, USA. We are grateful to Dr John B. Pike, The Upjohn Company, Kalamazoo, Mich. USA for the PGs.

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more Said *et al* (1974) did not document any effect of calibrating doses of PGs on the assay tissues.

In the present experiments we showed effect of calibrating doses of 0.5–2 ng/ml of PGE and PGF_{2α} (Fig. 3) but we could not detect any change in tissue tension during acute hypoxia. Effects caused by hypoxia *per se* was prevented by the oxygenator which maintained PO₂ above 500 mmHg in the blood superfusing the assay tissues.

Although we found no trace of PGs in the blood stream we could not rule out the possibility that hypoxia induced PG synthesis in the lungs. PGs could then accumulate in the tissue, locally influence vascular smooth muscle tone and subsequently be inactivated in the pulmonary circulation (Piper Vane and Wyllie 1970), without appearing in the venous effluent. To test this hypothesis lung tissue content of PGs of the F-series was measured by radioimmunoassay. The results of these measurements showed that in lungs exposed to alveolar hypoxia and with marked pressor responses, there was no increase in the tissue content of F prostaglandins. The tissue concentrations observed are similar to the low levels of PGF_{2α} reported earlier for rat lung tissue (Karim, Hillier and Devlin 1969).

Our results are thus in apparent conflict with the findings of Said and coworkers (1977). In addition to those already mentioned there are several reasons, however, why their work is difficult to assess. 1) They used a very low flow for perfusion of cat lungs (10 ml/min). 2) The basal level of P_{PA} was not presented and the reported responses to hypoxia were small (they considered a pressor response of 1 mmHg satisfying, but even this was not achieved in 8 of 32 expts.) 3) They used a perfusate of Krebs-dextran solution or a mixture of blood and Krebs-dextran. Recently we have found that any perfusate containing dextran cause spontaneous PG-synthesis in isolated lungs. After perfusion for some time with dextran solution there is a progressive release of substances with PGE-like activity in the situation stimuli which are without any effect in lungs perfused without dextran, now release great amounts of PG-like activity in addition to the basal release (Wiberg, Scott and Vaage 1977, Vaage *et al* 1977a).

In the present series of expts. an increased pulmonary synthesis of PGs with relative complete inactivation might theoretically escape detection. Several observations, however, make such an event highly unlikely. 1) Also PG-metabolites do to some extent cause constriction of the assay tissues (Crutchley and Piper 1975). 2) During pulmonary PG-synthesis unstable intermediates in PG-synthesis are released (Palmer *et al* 1973). These intermediates would partly have been converted to PGs of the H and F-series at the time when venous effluent blood reaches the assay tissues (Palmer *et al* 1973). 3) The radioimmunoassay results did not indicate any increase in PG-synthesis during hypoxia.

Kadowitz *et al* (1975) have reported that some synthesis of vasodilatory PGs is continuously going on in normal lungs. They found that indomethacin and meclofenamate, two structurally dissimilar inhibitors of PG-synthesis, increased pulmonary vascular resistance in dogs. These observations led to the suggestion that under resting conditions synthesis of vasodilatory PGs might be important for the maintenance of the pulmonary circulation in a dilated state. Such a background or basal production of PGs might as well play a role in setting the limits for any vasoconstrictor response to hypoxia. Inhibitors of PG-synthesis may act by preventing this modulating action of PGs with vasodilatory effect.

or smooth muscle, thus giving an increased pressor response to acute alveolar hypoxia (Björntorp and Hauge 1975 Tyler *et al.* 1975 Weir *et al.* 1976 a, Hales *et al.* 1977 *et al.* 1975). This modulating action is rather small and has no relevance in elucidating mechanism of the vasoconstrictor response to alveolar hypoxia.

Present results show that the pulmonary vasoconstrictor response to ventilation is not mediated by stimulation of PG synthesis.

It is interesting to note that the generous gift of Professor Laurence Levine, Brandeis University, W. Mass., USA. We are grateful to Dr John E. Pike, The Upjohn Company, Kalamazoo, Mich. USA, in gift of PGE₁.

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Effect of adenosine, adenosine analogues and drugs inhibiting adenosine inactivation on lipolysis in rat fat cells

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Abstract

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It is suggested that adenosine may be a physiologically important modulator of lipolysis. In the study it was found that adenosine inhibited lipolysis stimulated by low ($0.03 \mu\text{M}$) concentrations of norepinephrine (NA). Lipolysis stimulated by higher concentrations (0.3 and $3 \mu\text{M}$) of NA was inhibited to some or not at all. Theophylline (1 mM)-induced lipolysis was inhibited by adenosine ($\text{IC}_{50} \sim 10 \mu\text{M}$). One of theophylline-induced lipolysis was tested for several analogues of adenosine. Some N^6 -substituted analogues and 2-Ci-adenosine are more potent inhibitors. Adenosine nucleotides (ATP, ADP) were about equipotent with adenosine. Several adenosine analogues, including its breakdown products, were considerably less potent or ineffective. None of the analogues tested inhibited the action of a β -adrenergic agonist, isoproterenol, which inhibits the uptake of adenosine into cells, caused light enhancement of the catecholaminergic effect of adenosine. None of the analogues inhibited the action of adenosine. It is concluded that adenosine can inhibit lipolysis due to low "physiological" concentrations of norepinephrine and of low concentrations of theophylline via an action on a receptor structure at the cell surface. Such evidence structural specificity.

Adenosine is formed during incubation of isolated rat fat cells *in vitro* (Schwabe *et al.* 1973), and the release of adenosine-like material from the isolated canine subcutaneous adipose tissue following sympathetic nerve stimulation was recently reported (Fredholm 1976). *In vivo* conditions adenosine is a good inhibitor of lipolysis induced by sympathetic stimulation (Fredholm and Sollevi 1977). Furthermore, dipyridamol, a known inhibitor of adenosine inactivation (*e.g.* Schrader *et al.* 1972) was found to inhibit lipolysis induced by sympathetic nerve stimulation (Fredholm and Sollevi 1977). These findings are in line with the opinion that adenosine might act as a physiologically important modulator of lipolysis.

The interpretation of the results is, however, complicated by the published findings of various adenosine analogues: ATP-adenosine 5'-triphosphate; ADP-adenosine 5'-diphosphate; AMP-adenosine 5'-monophosphate; cyclic AMP-adenosine 3',5'-cyclic monophosphate; AMP (NH₂pp-adenosyl)-1- α -naphthylphosphonate; ADPCP- α , β -methyleneadenosine 5'-diphosphate; EHNA-erythro-2-(2-hydroxy-3-oxoethyl) adenosine.

adenosine effects in isolated rat adipocytes. There is complete agreement in the literature that adenosine and several analogs including the phenylisopropyladenosine are inhibitors of cyclic AMP accumulation induced by catecholamines (Fain 1973 a, b, c, *et al* 1973 Stock and Prikop 1974 Hjelm Dahl and Fredholm 1976 a). On the other hand, adenosine is remarkably ineffective as an inhibitor of lipolysis (Fain 1973 a, b, c, Hjelm Dahl and Fredholm 1976 a). As suggested by Fain (1973 b) this apparent discrepancy between the effects on cyclic AMP and the effects on lipolysis might be explained if the pool of cyclic AMP involved in turning on lipolysis is small and not in equilibrium with the bulk of the cyclic AMP in the cells. In isolated rat fat cells we recently reported the relationship between cyclic AMP and lipolysis (Hjelm Dahl and Fredholm 1977) found that elevations of cyclic AMP by only 2-3 pmol/10⁶ cells was necessary for half maximal stimulation of lipolysis. Since the basal cyclic AMP content is on average 4 pmol/10⁶ cells this represents only a 50 per cent increase in cyclic AMP. We recently found that under *in situ* conditions maximal lipolysis induced by sympathetic nerve stimulation was associated with a 50% increase in tissue cyclic AMP content (Hjelm Dahl, Belfrage and Blaschke 1977).

The effectiveness of adenosine as an antilipolytic agent *in vivo* might therefore be limited by the fact that the cyclic AMP levels *in vivo* are low. Conversely the relative ineffectiveness of adenosine as an antilipolytic agent *in vitro* may be due to the fact that the *in vitro* experiments commonly employ maximally effective concentrations of catecholamines which produce several fold increases in cyclic AMP.

From the arguments summarized above it was considered important to reconfirm the effect of adenosine on lipolysis caused by submaximal concentrations of lipolytic agents. For this purpose low concentrations of noradrenaline and low concentrations of theophylline were chosen. In addition we have studied the effect of a number of analogues of adenosine and of some substances known to interfere with the disposition of adenosine.

Materials and Methods

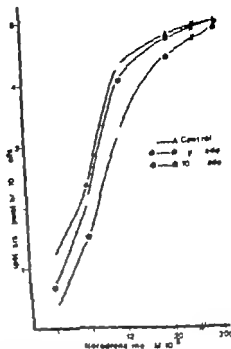
Experimental

Male Sprague Dawley rats (Anticimex) weighing 160-220 g were used. Adipocytes were isolated according to Rodbell (1964) as previously described (Fredholm and Hjelm Dahl 1976 a). Cell concentration was 30-80 × 10⁶ cells/ml. The cells were preincubated for 10 min with theophylline. Lipolysis was initiated by the addition of theophylline (final concentration 0.1 mM) or noradrenaline (final concentration 0.03-3 μM). In the latter case 10 μg ascorbic acid was included per ml of the medium. 1 h after the addition of the lipolytic drug 0.2 ml medium was deproteinized by ZnSO₄ precipitation and glycerol determined according to La Belle and Tibbling (1966).

Cyclic AMP was determined by a competitive binding assay (Brown *et al* 1971) in 10 sec incubations, withdrawn 5 min after the addition of the lipolytic drug pipetted into 0.5 ml ice-cold perchloric acid. After neutralization with 4 M KOH and 1 M Tris-base (pH 7.8), cyclic AMP was extracted in 50 μl aliquots of the supernatant. 85-100 per cent of the binding material was removed by phosphodiesterase (Boehringer Mannheim). Purification of the samples on Dowex 50-4 (100-200 mesh) ion exchange resin was not necessary since the values obtained were identical with those found without purification after correction for losses during purification.

Chemicals

Crude bacterial collagenase (from *Clostridium histolyticum*) was obtained from Worthington (Freehold, NJ, USA). Theophylline was used as the ethylenediamine salt (Oxyphylline® Astra, Södertälje, Sweden). Serum albumin (Fraction V), L-Noradrenaline hydrochloride, ATP, ADP, AMP, Adenosine, 8-bromo-adenosine, 2-chloro-adenosine were obtained from Sigma (St. Louis, USA).



1. The effect of adenosine on the lipolytic response to noradrenaline. Means of 2-4 determinations \pm 2 separate expts. (sept. 1, 67 000 cells/ml, 1.41 000 cells/ml). The cells were pretreated with 8 μ M ascorbic acid and adenosine at the concentrations indicated in the buffer for 10 min before doses of noradrenaline.

ypomethine from Merck (Darmstadt, BRD), nicotinic acid adenylsuccinate phosphate from Boehringer Mannheim (BRD), α , β -methyleneadenosine 3'-diphosphate (AOPCP) from PL Biochemicals (Milwaukee, WI), N^6 -phenylisopropyladenosine (PIA) and N^6 -c-cholerylladenosine were kind gifts from Dr. H. Z. Boeckler, Mannheim BRD. N^6 -(Δ -isopentyl)adenosine was kind gift of Dr. Bruce Hacker, NY, USA, and was dissolved in dimethylsulfoxide before use. N^6 -(3-hydroxy-3-methylbutyl)adenosine and N^6 -butyladenosine were kind gifts from Dr. H. Vorbrüggen, Schering AG (Berlin, FRG). The adenosine deaminase inhibitor α -methyl-9-(3-hydroxy-3-methyl)adenosine hydrochloride (EHNA) is from Wellcome Res. Labs. (Research Triangle Park, USA). Dibutyryl was gift from Dr. N. Brock, Würzburg (BRD). Dipyrindol was gift from Boehringer Ingelheim (BRD). Papaverine hydrochloride was obtained from ACO (Sweden). 3-methyl-1-methyl xanthine was obtained from Aldrich (Milwaukee, USA). Other chemicals were obtained from ordinary commercial sources.

Results

In agreement with previous findings (see Introduction) adenosine did not cause any significant inhibition of lipolysis induced by 3 μ M NA. Lipolysis induced by 0.3 μ M NA was inhibited by only 10% at 100 μ M concentrations, while lipolysis induced by 0.03 μ M NA was inhibited more than 50% already at 1 μ M adenosine in the medium (Fig. 1 a). Very similar results were obtained with phenylisopropyladenosine (not shown).

Corresponding cyclic AMP values are shown in Table I. Already at 0.3 μ M noradrenaline a essentially maximal cyclic AMP response was obtained. In the presence of adenosine (10 μ M) noradrenaline caused small, statistically not significant, increases in cyclic AMP in agreement with previous results (Pain 1973 a, Schwabe *et al.* 1973, Hjemdahl and Fredholm 1976 a).

The antilipolytic effect of adenosine was more pronounced at low concentrations of the-

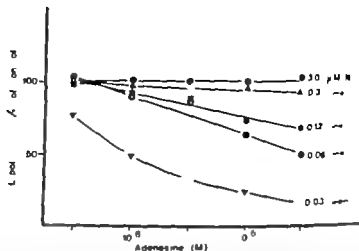


Fig. 1b. Inhibition of lipolysis by adenosine. Data were obtained from the same 2 expts. Results are expressed as per cent of the lipolysis induced by the corresponding concentration of noradrenaline in the absence of adenosine. Statistical hypothesis were tested by student's *t*-test. When lipolysis is activated by $0.03 \mu\text{M NA}$ significant inhibition ($p < 0.01$ or better) was obtained already with $0.3 \mu\text{M adenosine}$. $0.06 \mu\text{M NA}$ significant inhibition ($p < 0.001$) with $1 \mu\text{M adenosine}$ and higher. By $0.12 \mu\text{M NA}$ significant inhibition ($p < 0.05$) with $3 \mu\text{M adenosine}$ by $0.3 \mu\text{M NA}$ significant inhibition ($p < 0.01$) of lipolysis required $30 \mu\text{M adenosine}$.

phylline than at high (Fig. 2). The same was found for another methylxanthine, isobutylmethylxanthine (MIX) (Fig. 2).

The effect of adenosine and several adenosine analogues on lipolysis stimulated by isobutylmethylxanthine is shown in Table II. It can be seen from this table that the *N*-substituted adenosine analogues namely phenylisopropyl-adenosine, cyclohexylallyl adenosine and 1-hydroxy-3-methyl butyl adenosine as well as 2-Cl-adenosine are more potent than adenosine. A fourth *N*-substituted analog, $\nabla 2$ isopentyl adenosine is slightly less potent and the 3-butenyl butyldiene-adenosine, is practically devoid of activity. 8-bromoadenosine, 2-deoxyadenosine, inosine, adenine, and EHNA similarly have a very low activity. AMP and ADP are roughly equipotent with adenosine, while the ADP analog AOPCP is much less active. ATP and the analogue AMP(NH)PP are less active than adenosine.

Thus, some adenosine analogues are more potent than the parent drug. One possible explanation for this finding is that they are less readily taken up and/or metabolized than

TABLE I Cyclic AMP in fat cells treated with noradrenaline with and without adenosine and theophylline. Results are shown as means \pm S.E. The number of incubation is shown within parentheses.

	Cyclic AMP pmol 10^{-6} cells/5 min		
	No adenosine	$10 \mu\text{M adenosine}$	
Control	4.0 ± 0.9 (18)	4.2 ± 2.2 (10)	N.S.
Noradrenaline			
$0.03 \mu\text{M}$	5.0 ± 1.7 (17)	4.6 ± 2.4 (10)	N.S.
$0.3 \mu\text{M}$	10.0 ± 1.3 (18)	5.1 ± 2.2 (7)	$p < 0.001$
$3.0 \mu\text{M}$	11.5 ± 1.4 (17)	5.6 ± 3.2 (8)	$p < 0.001$
$30 \mu\text{M}$	9.0 ± 1.2	—	
Noradrenaline $3 \mu\text{M}$ + Theophylline 1 mM	259 ± 30 (24)	41 ± 15 (6)	$p < 0.001$

Inhibition by adenosine, analogues and related compounds of theophylline (1 mM) induced lipolysis. IC_{50} values were determined from the linear portions of the log-dose response curves obtained from quadruplicate determinations in at least two separate experiments.

	IC_{50} (μ M)	Lipolysis in 10 ⁻⁵ M of control at
theophylline	85	44
adrenaline	10	33
	9	44
	24	72
7,7-PP	35	76
7,7	—	106
2'		
2'-deoxyadenosine	0.3	7
adenosine	0.9	8
hydroxy-3-methylthio- adenosine	4	17
hydroxy-5-thio-adenosine	8.5	47
guanylateadenosine	20	64
2'-deoxyadenosine	—	74 ¹
2'-thioadenosine	—	90
2'	—	97
2'-thioadenosine	—	86
2'	—	103
2A	—	84
drugs		
acp	—	105
nicotine	—	106
propranolol	—	102

¹Actual inhibition (72%) as produced at 3 μ M.

amine. In order to test this possibility the 3 inhibitors of adenosine degradation, diltiazem, diltazep and papaverine were used. The results show that these drugs cause a 40% enhancement of the antilipolytic effect of adenosine, but the shift is a minor one (Fig. 3).

Another group of analogues appeared to be much less potent than the parent drug. The possibility existed that these drugs were partial agonists and hence could act to block the actions of adenosine. This possibility was tested. However none of the drugs could inhibit the antilipolytic effect of 10 μ M adenosine. Moreover 2-deoxyadenosine, which was virtually ineffective as an antilipolytic drug when used alone, inhibited lipolysis in the presence of isoproterenol by 50 per cent at a concentration of 50 μ M.

Discussion

The present results demonstrate that adenosine is antilipolytic *in vitro* provided that the concentration of the lipolytic drug is kept low. With the lowest dose of noradrenaline (0.03

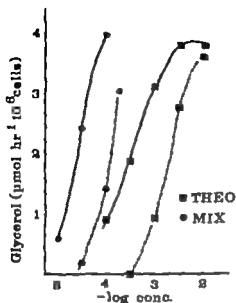


Fig. 2

Fig. 2. Lipolytic effect of theophylline (■) and 3-isobutyl-1-methylxanthine, MIX, (●) in the presence of adenosine 10 μ M. The cells (46 000/ml) were preincubated for 10 min before the addition of the methylxanthines and adenosine. Means of triplicate determinations.

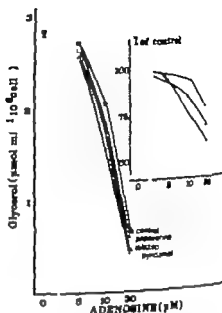


Fig. 3

Fig. 3. Effect of increasing doses of adenosine in the presence of 30 μ M papaverine (■), 30 μ M dilazep (□) and 3 μ M dipyrindamol (▽) on lipolysis induced by 1 mM theophylline. Mean of duplicate determinations from separate expts. (expt. 1: 46 000 cells/ml, expt. 2: 42 000 cells/ml). Inset: lipolysis in the presence of papaverine, dilazep and dipyrindamol in the presence of adenosine alone.

μ M) or with low doses of methylxanthines (0.3 mM theophylline, 0.03 mM MIX) adenosine caused an essentially complete inhibition in a concentration of 10 μ M.

When higher doses of the methylxanthines were used the antilipolytic effect of adenosine decreased as would be expected from an essentially competitive inhibition. On the other hand, when high concentrations of noradrenaline (1 μ M or above) were used to stimulate lipolysis even very high concentrations of adenosine did not inhibit lipolysis. This is in agreement with the results of Fain *et al.* (1972) and of Stock and Prilop (1974). On the other hand, Ebert and Schwabe (1973) found a decreased maximal lipolytic response to noradrenaline by adenosine. We have no ready explanation for this discrepancy.

It has been shown repeatedly that adenosine inhibits noradrenaline-induced cyclic AMP elevations in fat cells to a much larger degree than lipolysis (Schwabe *et al.* 1973, Fain 1972, Stock and Prilop 1974, Hjemdahl and Fredholm 1976a). This might be taken as evidence for a dissociation between cyclic AMP and lipolysis (e.g. Stock and Prilop 1974). However, the relationship between drug-induced cyclic AMP elevations and drug-induced lipolysis is not linear. Very small elevations of cyclic AMP are necessary to produce a maximally maximal lipolysis (Hjemdahl and Fredholm 1976a). It is possible that the very small increases in cyclic AMP due to noradrenaline in the presence of adenosine (Schwabe 1973, Hjemdahl and Fredholm 1976a, Table I present study) is sufficient to explain the induced, essentially maximal, lipolysis. It is generally assumed that cyclic AMP levels

sis via activation of protein kinase that activates a hormone-sensitive lipase (see Stein *et al.* 1976). Thus, cyclic AMP bound to the regulatory subunit of protein kinase may be a functionally important part of the nucleotide. In agreement with such a view it was also found that the bound form of cyclic nucleotide changes over a much smaller range than the free, possibly less important form of the nucleotide (Terasaki and Brooker 1977). Inhibitors of cellular adenosine uptake such as dipyrindamol, dflazep and papaverine do inhibit the actions of adenosine on lipolysis and cyclic AMP accumulation (Fain *et al.*

Ebert and Schwabe 1973, present data). If anything a slight potentiation of the action of adenosine was seen. This clearly suggests that adenosine acts on the outer surface of the cell. These findings also tend to rule out inhibition of protein kinase (*e.g.* Kariya and Field 1973) as an important mechanism in the antilipolytic action of adenosine.

The bulk of the evidence thus suggests the existence of a surface adenosine-receptor coupled to the fat cell adenylate cyclase by some unknown mechanism. In order to study the structural requirements at this receptor site a number of analogues were tested. Of the naturally occurring analogues AMP and ADP were approximately equipotent with adenosine. ATP was less potent, while inosine and adenine were essentially devoid of activity. Interestingly the two "stable" nucleotide analogues AMP(NH)PP and AOPCP were considerably less active than their unstable natural counterparts, suggesting that breakdown to adenosine is essential for activity. The finding that breakdown products of adenosine are essentially devoid of activity is in agreement with earlier results (Fain *et al.* 1972, Ebert and Schwabe 1973).

In agreement with earlier findings we found that N^6 -phenylisopropyladenosine was more potent than its parent compound (Fain *et al.* 1972, Ebert and Schwabe 1973). 2-chloroadenosine was also almost ten times as potent as adenosine. Since these compounds are identical to the action of adenosine deaminase the high activity could be due to a lower rate of degradation. However, adenosine in the presence of EHNA, a very potent inhibitor of adenosine deaminase, or in the presence of either of three inhibitors of adenosine uptake, was still considerably less potent than 2-chloroadenosine and PIA, rendering this possibility unlikely. Furthermore, adenosine deaminase activity in fat cells is low (Fredholm, unpublished). Thus, certain adenosine analogues may have a higher affinity than adenosine to the proposed receptor while other analogues have a lower affinity and/or lower intrinsic activity. The latter possibility was particularly interesting since a partial agonist with low intrinsic activity may be used as an inhibitor. Therefore the analogues with low activity were tested in combination with adenosine. However, none of these compounds were found to significantly antagonize the effect of adenosine. It is reasonable to assume therefore that their low biological activity is due to low affinity to the receptor rather than to low intrinsic activity.

Under physiological conditions noradrenaline appears to be lipolytic in very low concentrations. Thus, when noradrenaline is given by i.v. infusion essentially maximal rates of lipolysis are seen when calculated plasma concentrations are as low as $0.02 \mu\text{M}$ (Hjemdahl and Fredholm 1976 b). Also during nerve stimulation the effective noradrenaline concentrations are quite low since most fat cells are not in direct contact with nerve terminals (Fredholm 1970, Belkridge *et al.* 1977). Therefore the present results showing an antilipolytic

effect of adenosine provided that the concentration of noradrenaline is in the physiological range offers support for the possibility that adenosine may play a physiological role as a modulator of lipolysis (Fredholm 1976, Fredholm and Sollevi 1977).

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Prostaglandin-mediated inhibition of noradrenaline release: II. Dual mechanism behind its frequency-dependence

By

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Abstract

WENGMALM, Å. Prostaglandin-mediated inhibition of noradrenaline release. II. Dual mechanism behind its frequency-dependence. *Acta physiol. scand.* 1978. 102. 199-204.

Experimentally innervated, isolated rabbit hearts were perfused according to Langendorff and the nerves stimulated at 2, 5 or 10 Hz by equally long trains of pulses. The outflow of prostaglandin-like substance (PLS) and of noradrenaline (NA), induced by the nerve stimulations, were followed. The sensitivity to process of NA release to exogenous PGE_2 ($2-6 \cdot 10^{-6}$ M) at 2, 5 and 10 Hz was studied. The outflow of PLS is found to be frequency-dependent, being greater at 2 Hz than at higher discharge rates, while outflow of NA is similar at the different frequencies. The inhibitory action of PGE_2 on the NA release process is more pronounced at 2 than at 10 Hz. It is concluded that the frequency-dependence of endogenous PGE_2 -mediated inhibition of the release of NA from discharging sympathetic nerves is based on independent, frequency-related mechanisms: a) higher synaptic rate of PLS/impulse, and b) low pre-synaptic sensitivity of the process of NA release, at low compared to higher impulse frequencies.

Endogenous prostaglandins of the E series (PGE) have an inhibitory action on the process of release of sympathetic neurotransmitter (noradrenaline, NA) from depolarising adrenergic nerves in perfused organs from various species (Sjörnsten and Wengmalm 1971, Hedqvist, Sjöström and Wengmalm 1971, Swedin 1971, Chanh, Junstad and Wengmalm 1972, errors, Moncada and Vane 1973, Starke and Montel 1973). There is evidence that this action of endogenous PGE is present also in intact animals (Sjöström 1971, Junstad and Wengmalm 1972, Fredholm and Hedqvist 1975). The mechanism by which PGE operates to depress the release of NA from depolarising nerves is incompletely known. Hedqvist (1970) demonstrated that the inhibitory action elicited by exogenous PGE_2 on NA release from the perfused stimulated cat spleen was counteracted by increased calcium concentrations in the perfusing medium, and this observation was confirmed in the guinea pig vas deferens (Sjöström 1973 a). Later the capacity of endogenous PG to counteract the release of NA was demonstrated to relate inversely to the depolarising frequency in the nerves (Junstad and Wengmalm 1973, Sjöström 1973 b). The latter observation, suggesting that the inhibitory action of endogenous PGE is most pronounced at low depolarising frequencies (1-2 Hz),

supports the "physiological" significance of the PGE mediated inhibition. The investigation was therefore undertaken to study the mechanism behind this dependence. The method chosen was to investigate, first, the outflow of PLS (prostaglandin-like substances) from the heart at different impulse frequencies in its sympathetic and second the sensitivity of the NA release process to PGE at different impulse frequencies.

Methods

a. Rabbit heart perfusion

Rabbits of mixed strains and sexes were used for the study. The weight of the animals varied between 2 and 4 kg. After a blow on the head, the animal was exsanguinated by cutting the left carotid artery. The heart was dissected free with its bilateral sympathetic nerve supply kept intact (Huković and Marchal 1967). It was subsequently transferred to the perfusion apparatus, where it was perfused according to Lundquist (1967) with Tyrode solution of the following composition (in mM): NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaHCO₃ 24, NaH₂PO₄ 0.4, glucose 5.6. The solution was aerated continuously with 5% CO₂ in O₂. The pH of the solution was 7.4-7.5 and its temperature was kept at 37°C. The sympathetic nerves were pulled through plastic tubes with circumferential platinum rings serving as electrodes. The nerves were connected to a Grass Model 348 stimulator. The nerves were stimulated with trains of 1000 rectangular pulses, 25 V in height, and of 1 ms duration, delivered at 2, 5 or 10 Hz.

In one series the effect of varying the frequency of the sympathetic nerve stimulation on the outflow of PLS from the heart was analysed. Each heart was stimulated three times, at 2, 5 and 10 Hz respectively, the stimulations being separated by a resting interval of 15 min. The venous effluent from the heart was collected from the beginning of each stimulation and for the following 10 min (1 Hz, 5 min; 5 or 10 Hz, 4 min). The sequence of stimulation frequencies was varied between the experiments so that one third of the series the experiments were started at 2 Hz, in one third at 5 Hz and in one third at 10 Hz.

In another series the capacity of exogenous PGE to inhibit the outflow of NA from the heart at different frequencies of sympathetic nerve stimulation, was studied. The hearts, dissected and perfused as above, were stimulated twice, with a 15 min interval, at either 2, 5 or 10 Hz, with trains of 1000 pulses. PGE was infused through a cannula above the aorta into the solution perfusing the heart, from the beginning of the first effluent collection period until the end of the experiment. The final concentration range of PGE was 10^{-6} to 10^{-8} M. NA in the effluent was analysed using the tritiated tryptamine method.

b. Assay of PLS

The venous effluent from the heart was collected on ice and within 1 h after the collection PLS was extracted on 7 g columns of Amberlite XAD-2. PLS was eluted with ethanol. After evaporation to dryness the residue was dissolved in 1 ml of saline. The biological activity of the dissolved residue was tested against known amounts of PGE₂ on the superfused rat stomach strip (Vane 1957). The assay organ was a 5 ml organ bath containing Tyrode solution of the composition given above, to which was added 10⁻⁶ M of amine (7 × 10⁻⁶ M), propranolol (8 × 10⁻⁶ M), tropine (10⁻⁶ M), methysergide (6 × 10⁻⁶ M), phenylephrine (7 × 10⁻⁶ M), in order to block activity in the strip due to the presence in the bath of noradrenaline, acetylcholine, serotonin and histamine. Usually 1/2 of the lipid extract from the heart was tested on the stomach strip. Since the assay organ was sensitive to prostaglandins of the E-series, amounts of 1-2 ng, an outflow of prostaglandin-like substances in the effluent during the perfusion period of less than 5 ng could be detected. Each lipid extract was tested twice and the intra-assay variation in the samples in repeated assays was usually less than 15%. The recovery of the prostaglandin extraction procedure was checked in every extraction series by the use of external standards, and the results given for outflows of PLS have been corrected using the actual recovery (72 ± 5%).

c. Calculations

The outflow of PLS was calculated as follows. After bioassay against PGE₂ and volume correction the PLS content of a sample was corrected for the actual recovery of PGE₂, estimated using the external standard. The outflow of PLS is presented as the mean ± S.E. of the outflow of PLS at 2, 5 or 10 Hz, respectively. The outflow figures are also presented as outflow of PLS/min, by dividing the total outflow by the duration of the effluent collection period. Finally the outflow figures are presented as outflow of PLS/impulse, by subtracting from the total outflow the estimated basal outflow during the respective effluent collection periods.

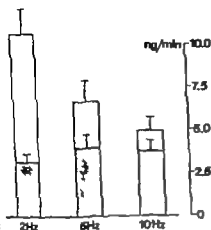


Fig. 1

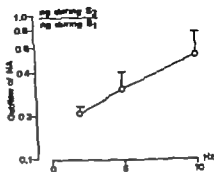


Fig. 2

1. Outflow of PLS from the sympathetically stimulated rabbit heart. The total height of the columns is the outflow of PLS during 1 000 pulse trains of nerve stimulation at 2 Hz, 5 Hz, or 10 Hz (left axis), and the striped columns indicate the outflow of PLS/min during the respective frequencies of stimulation (right ordinate). All values are presented as mean \pm S.E.

2. Effect of PGE₂ ($2-6 \times 10^{-6}$ M) on the outflow of NA from the isolated rabbit heart, sympathetically stimulated at 2 Hz, 5 Hz, or 10 Hz. The values given represent the ratio between the outflows of NA during successive nerve stimulations (1 000 pulses), delivered at the frequencies indicated. Values given as \pm S.E.

3. Capacity of exogenous PGE₂ to inhibit the release of NA during nerve stimulation is expressed as ratio between the NA in the effluent during the second (PGE₂ present) and the first periods of stimulation and effluent collection.

Results

Outflow of PLS at different frequencies

Stimulation of the sympathetic nerves to the heart at 2 Hz with trains of 1 000 pulses elicited an increase in heart rate (29 ± 4 beats/min) and contractile force. An outflow of NA appeared in the venous effluent, amounting to 179 ± 30 ng, during the stimulation period. The outflow of PLS was markedly increased, corresponding to almost 30 ng of PGE₂ (Fig. 1) during the same effluent collection period. After the basal outflow of PLS had been subtracted, the outflow of PLS induced by the 2 Hz sympathetic nerve stimulation corresponded to 19 ± 4 pg PGE₂/imp.

At 5 Hz the mechanical response of the heart to trains of 1 000 pulses was more pronounced, the heart rate being increased by 51 ± 5 beats/min. The outflow of NA was, however, of the same magnitude as that obtained at 2 Hz (183 ± 39 ng). The total outflow of PLS at 5 Hz was about 2/3 of that obtained at 2 Hz (Fig. 1). Estimated as outflow of PLS/min it was, however, the same as that at 2 Hz. The net outflow of PLS at 5 Hz (total-basal) corresponded to 12 ± 3 pg PGE₂/imp.

The 10 Hz sympathetic nerve stimulation elicited the most marked mechanical response of the heart inducing an increase in heart rate of 70 ± 9 beats/min. The overflow of NA was slightly but insignificantly higher (212 ± 52 ng) than at 2 Hz or 5 Hz. The total outflow of PLS decreased further as compared to 2 Hz and 5 Hz. However, expressed in ng/10 min, the outflow of PLS at 10 Hz was of the same order as at 2 or 5 Hz (Fig. 1). The outflow of PLS at 10 Hz (total-basal) corresponded to 11 ± 3 pg PGE_2/min .

b Sensitivity of NA release to PGE_2 at different frequencies

Stimulation of the sympathetic nerves at 2 Hz (1 000 pulses) was followed by an overflow of NA of 710 ± 295 ng, heart rate increased 30 ± 6 (41 \pm 8 beats/min). A second stimulation at 2 Hz in the presence of PGE_2 ($2-6 \cdot 10^{-4}$ M) elicited a release of NA amounting to only about 20% (155 ± 75 ng) of that obtained during the first nerve stimulation (Fig. 2). The chronotropic response was unaffected, or even slightly increased (40 ± 7 46 ± 6 beats/min) during the second nerve stimulation, the ratio between the chronotropic responses during the second and first nerve stimulation being 1.20 ± 0.07 .

At 5 Hz the overflow of NA during the first nerve stimulation was 495 ± 90 ng; heart rate increased 42 ± 14 (51 \pm 11 beats/min). PGE_2 ($2-3 \cdot 10^{-4}$ M) inhibited the overflow of NA during the second nerve stimulation at 5 Hz to about 30% of the control (Fig. 2). The chronotropic response (43 ± 15 -51 ± 14 beats/min) was unaffected by the presence of PGE_2 during the second nerve stimulation.

When the sympathetic nerves were stimulated at 10 Hz, $1\,040 \pm 475$ ng of NA appeared in the effluent; heart rate increased 57 ± 3 (84 \pm 4 beats/min). PGE_2 ($2-4 \cdot 10^{-4}$ M) inhibited the overflow of NA during the second 10 Hz nerve stimulation to about 30% of the control (Fig. 2). The chronotropic response (48 ± 5 -64 ± 4 beats/min) was slightly smaller in the presence of PGE_2 during the second nerve stimulation.

Discussion

In the present study equally long trains of pulses stimulating the sympathetic nerve at 2, 5 or 10 Hz delivered to the isolated rabbit heart elicited the same overflow of NA into the venous effluent when delivered at 2, 5 or 10 Hz. In contrast the venous outflow of PLS varied with the frequency, being greater during the 2 Hz stimulation compared to 5 Hz and 10 Hz. The larger outflow of PLS at 2 Hz was apparently due to the longer effluent collection time at this frequency compared to 5 or 10 Hz, when expressed in relation to time there was no difference between the outflow figures of PLS at the different frequencies. The mechanism leading to this frequency-dependence is not obvious. It might be argued that since the different stimulations, at various frequencies but of the same number of pulses, gave rise to similar NA overflows, a stoichiometric relation would decide the amount of PLS synthesized per mole of NA. However, the actual concentration of NA at the PLS synthesis stimulation sites may have been considerably higher at the 10 Hz than at the 2 Hz stimulations in the present experiment and consequently PLS synthesis should have been more stimulated at the higher frequency. More plausible, then, are either of the alternatives that the PLS synthesis stimulation induced by sympathetic nerve stimulation is fully developed already at 2 Hz and that an increase

rate does not further elevate the degree of PLS synthesis stimulation, or alternatively there is a delay in the onset of PLS synthesis stimulation following nerve stimulation that affects short stimulation periods relatively more than longer ones. The sensitivity of the NA release to exogenous PGE varied also with the frequency of stimulation in the present study being considerably greater at 2 than at 10 Hz. Hedqvist and Stjärne (1973 a) reported that PGE, when inhibiting the release of NA, acts by competing with the availability of Ca^{++} . Furthermore, in the same paper Stjärne demonstrated a close relationship between the inhibitory effect of PGE and the calcium concentration in the medium on the neuronal NA release. In the light of these results the data, showing less inhibitory effect of PGE at increased discharge rates, may be explained as follows: increased depolarisation frequency in the sympathetic nerve elicits needed neuronal concentration of Ca^{++} available for the NA release. At a certain ratio of PGE the inhibitory action of this drug on the NA release process is less pronounced at higher than at lower Ca^{++} concentrations. The more pronounced inhibitory effect of PGE at 2 Hz compared to 10 Hz would thus be expected. In conclusion, the frequency-dependence of the inhibition of NA release from depolarising sympathetic nerves, induced by endogenous PGE, seems to be based on 2 independently frequency-related mechanisms, a) a greater outflow of PGE per nerve impulse at low compared to high discharge rates, and b) an increased sensitivity of the NA release process to the inhibitory action of PGE at low compared to high impulse frequencies.

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- VANE, J. R., A sensitive method for assay of 5-hydroxytryptamine. *Brit J Pharmacol* 1957 12 54

The spatial distribution of odour induced potentials in the olfactory bulb of char and trout (Salmonidae)

By

GEORG THORESEN

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Abstract

MESEN, G. The spatial distribution of odour induced potentials in the olfactory bulb of char and trout (Salmonidae). Acta physiol. scand. 1978. 102. 205-217

olar DC-recordings were made of the gross response from the olfactory bulb of char (*Salvelinus* spp. *Salmo alpinus* L.) and trout (*Salmo trutta* L.) during stimulation with different odours. The parameters studied were the magnitude and polarity of the slow potential shift, the amplitude of fast waves and their symmetrical waveform. Amino acids elicited the largest responses in the part of the bulb. Water containing "crude fish odour" caused the largest responses in the rostral bulb parts. The results demonstrate odour specific differences in the localization of the bulb response and the separate origin of the slow potential and the induced fast waves.

Key words: Odour stimulation, response specificity, spatial distribution, olfactory bulb, teleost.

vertebrate olfactory organ serves several purposes, including seeking for food and specific communication. In salmonid fishes the intraspecific communication may also pheromone detection important for the homing behaviour of the spawners (Nordeng & Solomon 1973).

adequate stimulants for the salmonid olfactory organ are predominantly amino acids (Berth & Sutterlin 1971) and related compounds and yet unidentified components of fish skin mucus (Døving, Enger and Nordeng 1973). Such components are suspected to be pheromones (Døving, Nordeng and Oakley 1974).

Anatomical investigations on the teleost olfactory system show a diversity in the central sections of olfactory bulb onto various parts of the brain (Sheldon 1912, Holmgren 1920). Anatomical and physiological studies on the olfactory tract of the burbot show that it is divided in bundles with different spectra of fiber diameters and conduction velocities (Enger and Gerson 1965).

From electrophysiological and anatomical work on mammals it is known that the olfactory bulb is functionally subdivided. Information on different odours is treated by distinct, if not necessarily exclusive parts of the bulb (Adrian 1951) involving partially parallel populations of secondary neurons (Pinching and Døving 1974). It is therefore

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GEORGE THOMASSEN

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Abstract

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Micro-DC-recordings were made of the gross responses from the olfactory bulb of char (*Salvelinus alpinus* L.) and trout (*Salmo trutta* L.) during stimulation with different odours. The following studies were: the magnitude and polarity of the slow potential shift, the amplitude of focal waves and their asymmetrical α - γ deflection. Amino acids elicited the largest responses in the part of the bulb. Water containing "crude fish odour" caused the largest responses in the rostral lateral parts. The results demonstrate odour specific differences in the localization of the bulb response and the separate origin of the slow potential and the induced waves.

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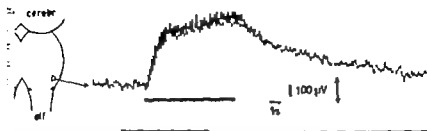
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The adequate stimulants for the salmonid olfactory organ are predominantly amino acids (Leite and Sotterlin 1971) and related compounds and yet unidentified components of fish skin mucus (Døving, Eger and Nordeng 1973). Such components are suspected to be pheromones (Døving, Nordeng and Oakley 1974).

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The general response characteristics illustrated by the DC recording of the response to 10^{-4} M of an odorant. The recording was made from the surface of the rostralateral corner of the left olfactory bulb. The marker pen trace (bottom) shows the switching of the stimulation device. The thick bar above the trace indicates the stimulation period. Note the asymmetry of the induced waves.

was not consecutive. The order of the stimuli was varied at random. The period of adaptation stimulation was at least 30 s or as long as necessary so that the previous response remained. Stimulation time was adjusted manually and adjusted for the time lag present due to the construction of the stimulation device.

Amplitude of responses

Amplitude shifts were measured as the mean deflection of the tracing at 3–4 s after the onset of the stimulus. Due to the general instability of the DC recording the measurements of the DC deflections are within $\pm 50 \mu\text{V}$. The AC-responses were measured as the mean amplitude of 3 recordings, from 3 to 5 s after the onset of the response, by means of a polar plotter. The polarity of any asymmetry occurring was noted. As a control on possible differences in physical conditions such as variable activity of the olfactory bulb surface and skinning effects, the spontaneous activity previous to each stimulus has been evaluated by the same method as the AC-response. The measurements of the AC signals showed a standard deviation of about $\pm 10 \mu\text{V}$ as judged from a great number of quantifications of the spontaneous activity.

Results

General response characteristics

Surface responses to olfactory stimulation had two main components as previously described by Orison (1959a and b), a sustained component or DC-potential shift and an oscillation hereafter referred to as "induced waves" (Adrian 1950). An example of response with the typical characteristics is shown in Fig. 1. With the stimulation system in these experiments the latency could not be determined. The response increased gradually during the first 2–3 s of stimulation. At this time a breaking point was often seen, after which the response stabilized and persisted as long as the stimulus was maintained for up to several tenths of seconds. When the stimulus was turned off the response declined roughly exponentially with a half-time of 3–10 s.

Typical DC potential shifts were monophasic deflections of the baseline, with a magnitude ranging from -250 to $-1000 \mu\text{V}$.

These waveforms the DC shift was disturbed by mechanical artifacts due to the switching of the stimulator and the contemporary transient flow change. These artifacts were short lasting, usually positive deflections of up to $100 \mu\text{V}$. Occurring simultaneously with the manual stimulation switching, they were easily detectable and did not interfere with the true responses. Positive deflections simultaneous with true stimulations and with rapid onset and ending were also seen. These deflections did not seem to be related to ionic concentrations. Similar reactions could be artificially produced by strongly stirred in the bath water instead of the usual aquarium water. They disappeared when the electrode was placed

likely that the bulbo-cerebral connections in the fish pointed out by Sædén (1971) and Holmgren (1970) are reflected in the functional organization of the fish olfactory bulb.

The aim of the present study was to find the spatial differentiation of the projections of the bulb to various odours. In this article the odour specific differences in the responses are described by examples of the responses to stimulation with some amino acid "fish odour" samples. A preliminary report of this work has been made (ThommSEN 1972).

Material and Methods

The material consists of two experimental series, one on the brown trout (*Salmo trutta* L.) and on the arctic char (*Salvelinus alpinus* syn. *Salmo alpinus* L.). The present study is based upon recordings from trout and III chars. The trout, weighing about 100 g, were caught by an electrical fishing apparatus in a local brook in Oslo. 16 of the chars were hatched in 1970 as offspring of a few parents caught only at their common spawning site in lake Lona in western Norway (Nordeng, personal communication). These chars were therefore presumed to be as genetically homogeneous as could be expected in an interbreeding population in nature. At the time of experiment they were about 6 years old and about 300 g. Two of the chars had been caught by net in the neighbouring lake Vangsvatn.

The fish was paralyzed with Tubocurarin (Nyco) 1 mg tubocurarin-chloride i.m. per 100 g. The paralyzed fish was wrapped in a wet sponge and secured in a Plexiglass form by steel rods and claws of a headholder. The gills were perfused with aquarium fresh water (Oslo city water) at 5-10 ml/min. The dorsal aspect of the olfactory bulbs was exposed. Each bulb was approximately 1 mm across and 1.5 mm in the char.

Recording. The potentials were picked up by glass capillaries filled with a physiological salt solution. Electrodes were inserted in electrode holders containing sintered Ag/AgCl. Electrode tip diameter from 20-100 μ m. The potential changes were fed to preamplifiers with frequency response DC-150 Hz and monitored on a pen recorder. All recordings were monopolar and the preparation was grounded through the indifferent electrode only which was placed on the chondrocranium just posterior to the opening in the skull.

Stimulation

The stimuli were presented from a polyethylene pipette through a continuous flow of aquarium water to the ipsilateral anterior nares and controlled by a 3-way stopcock. Nasal water flow was kept at 4-6 ml/min.

In this presentation the choice of stimuli has been limited to a) the following L- α -amino acids: glutamine, histidine, lysine, methionine, serine, tryptophan and tyrosine in the concentrations 10^{-4} M and b) crude odour samples from the same populations as the experimental animals. The "fish odour" samples were chosen in order to look for possible species- and population-specific differences in the responses as they were suspected to contain population specific pheromones (see Osseward 1971). The crude fish odour samples were tapped from the basins where the fishes were kept during the experiment. The concentrations were presented in half decade concentration steps from undiluted to below threshold concentrations. The concentrations of the "fish odours" were estimated in terms of the number of fishes per hour passing through it (fish/hour). The concentrations used ranged from 3.6 to 0.02 fish/hour. Glutamate "crude fish odour" were tested in all experiments.

Experimental procedure

Responses have been recorded from both olfactory bulbs stimulating the ipsilateral olfactory bulb. The recording electrodes were placed in 15 positions covering the dorsal surface of the bulb. The positions were recorded in each experiment. In all the chars the responses were recorded from 2-3 positions simultaneously one electrode was kept stationary serving as a control of stimulation and of the preparation.

The blood flow in the brain surface vessels was regularly inspected as a control on the circulation, especially during the manipulation of the electrodes.

Each kind of stimulus was presented in the order of ascending concentration, each concentration

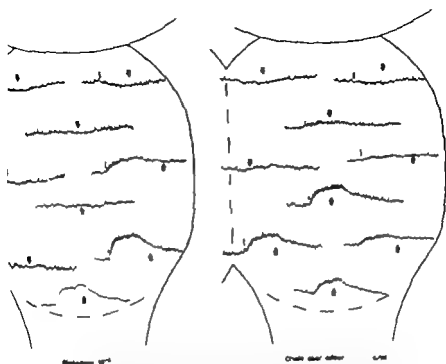


Fig. 2. Typical DC recordings of the response in selected positions on the dorsal side of the left olfactory bulb during odour stimulation of the epilateral olfactory epithelium with (a) glutamine, 10^{-4} M, "crude char odour" 0.6 fish g/l. The recordings are displayed at their respective recording positions on the outside of the left olfactory bulb. The solid arrows pointing at each recording are placed at the actual recording area. The width of the arrow shafts is 1 mm. The short vertical bars marking the onset of the responses are 100 μ V negative upwards.

The differences in polarity and magnitude of the DC potential shift as well as those of the induced

dorsal surface and in the center of the bulb was related to those recorded from the dorsal surface. These recordings were also interesting in view of the current ideas about the site of origin of the responses (see Discussion). Fig. 3 displays results from such an experiment. The electrode was pushed from the center of the dorsal surface of the olfactory bulb, vertically through the bulb until it touched the braincase bottom and withdrawn in 0.1 mm steps. Compression of the tissue during the penetration made the determination of the position of the electrode tip uncertain. The recordings, therefore, were only made during the withdrawal of the electrode.

The figure confirms the odour specific differentiation of the bulbar responses to glutamine and "crude char odour". It shows that the response to glutamine is far stronger at the ventral than central on the dorsal and in the center of the bulb while "crude char odour" tends to give a more uniform response distribution. In the center of the bulb all induced waves showed positive sharp peaks while the DC shift almost disappeared. The changes of polarity of the sharp peaks occurred approximately 0.3 mm from the ventral and dorsal surfaces

forward on the olfactory nerve. They were therefore not projections of the electroolfactogram. This phenomenon was regarded as an artifact, but being contemporary with the true odour stimulation, it with the measurements of the DC response and may in some recordings have produced a bias of $\pm 100 \mu V$.

The AC component of the recordings changed at stimulation from irregular spontaneous activity (amplitude range $20-90 \mu V$ peak to peak, into regular induced waves with a peak amplitude of up to $600 \mu V$ and frequency range of $3-6$ Hz. The induced waves had an asymmetrical waveform with sharp and blunt peaks. Spindle-like bursts of actions were rare. In some cases the induced waves showed alternating high and low amplitudes. These more sporadic phenomena have not been subject to analysis on the work.

Variability in response

Stimulation with a given odorant at a certain concentration induced responses which different recordings in different regions of the bulb. Other odorants elicited other patterns of response distribution. The most prominent differences in response distribution were found between any of the "crude fish odours" and any amino acid. The various features of the responses are illustrated in different ways in Fig. 2-7. Except for Fig. 12, all of the figures displays results from one preparation only.

Fig. 2, shows the qualitative differences in the surface responses to stimulation with an amino acid (glutamine) and a "crude fish (char) odour" at selected recording positions covering the dorsal surface of the olfactory bulb. The differences refer to the magnitude of the DC-shift, the relative amplitude of the induced waves and the polarity of the sharp peaks. The figure is composed of recordings from 4 consecutive char preparations with qualitatively and quantitatively similar responses in common control positions.

Glutamine gave a pronounced negative DC shift in the rostralateral half and a weak positive shift in the caudomedial half. The induced waves had largest amplitudes in the rostralateral half showing negative sharp peaks. They were nearly undetectable along the dorsal midline and weak with positive sharp peaks at the medial side. Thus the polarity of the sharp peaks followed that of the DC potential shift except in the caudolateral corner of the bulb where negative sharp peaks were superimposed on a positive DC shift. In this corner and in the extreme rostral end of the bulb the spatial difference in distribution of the AC-response and the DC-response was clearly seen.

The distribution of responses to "crude char odour" was different. The DC shift and the induced waves were largest in the rostral parts of the bulb. In the same region both a DC shift and the sharp peaks of the induced waves were negative. Also with this odorant a slight separation of the two response components, the DC shift and the induced waves, were seen. The largest induced waves were found a little more caudally than the greatest DC shift.

The recordings which were all made with two electrodes simultaneously showed that the sharp peaks of the induced waves always appeared exactly in phase or in counterphase throughout the entire olfactory bulb.

Repetition experiments were performed to see how the distribution of responses varied

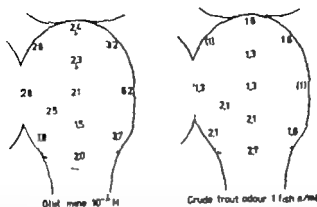


Fig. 5. Spatial distribution of the responses to glutamate, 10^{-4} M and "crude trout odour" 1 fish s/ml, on the left and right bulb of trout. The numbers give the amplitudes of the induced waves relative to that of the spontaneous activity. The + and - signs indicate the polarity of the sharp peaks. No sign indicates synchronous activity. (0) indicates that induced waves were not detected.

concentrations used, 10^{-4} M, the curves being approximately linear. The slopes were steepest at the rostral position and corresponding responses at higher concentrations were greatest at the lateral position. At the dorsal position the responses were weaker and of the opposite polarity. At the two recording sites along the dorsal midline of the bulb the responses were small and varied little with concentration. Similar results were also found for the other amino acids used.

Results from stimulation with "char odour" are less conclusive as the concentration was only 1% decade. Only at the rostral recording position the response magnitude was linearly related to stimulus concentration. At this position the results parallel those found for leucine at the lateral position. At the medial position the induced waves were weak and showed no obvious asymmetry.

Together the curves indicate that provided the stimulus and the recording position do not change, the magnitude of the DC shift is approximately proportional to the amplitude of the induced waves above the spontaneous activity.

As indicated in Fig. 5 there was an approximately linear relationship between the magnitude of the DC shift and the amplitude of the induced waves. This relationship is also illustrated in Fig. 6. In addition this figure shows in a quantified manner an example of the spatial mirror image in the magnitudes of the two response components also demonstrated in Fig. 2. Recordings from homologous positions in the ipsi- and the contra-lateral bulb during lateral stimulation of the olfactory epithelium showed that the spatial distribution of responses to any given stimulus were mirror images of each other and that the responses increased with increasing stimulus concentration in a similar manner in homologous positions. In reliance on this symmetry results from the right bulb have been presented in this article as if they were all obtained from the left. This is the case in Fig. 3 and 5 and for some recordings in Fig. 2.

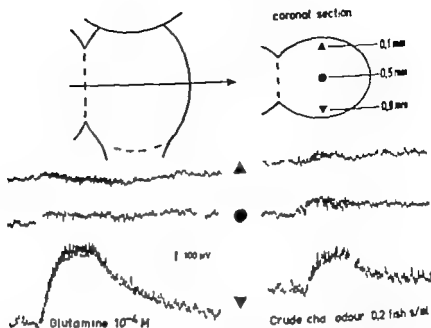


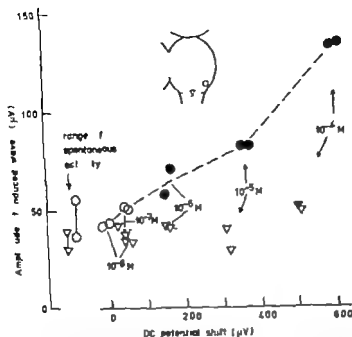
Fig. 3 Recordings from penetration vertically through the center of the olfactory bulb of a daf sketch on the top shows the approximate position of the electrode tip for 3 selected couples of record. Note the lack of significant DC shifts in the center and the changes in the polarity of the sharp peak.

using "crude char odour" just ventral to the center of the bulb when using glutamate stimulus. No histological investigation has been made in this study. The different recording positions can therefore not be exactly related to any specific layer of the olfactory bulb. In spite of the greater responses on the ventral side most of the recordings were therefore made from the dorsal surface of the bulb.

Measurements of the response amplitude have been made on a number of fish. Fig. 4 displays the results from AC recordings on a trout. The spatial variation in the induced waves during stimulation with glutamine and "crude trout odour" in a single preparation is shown. The responses have been quantified as the ratio of the peak to peak amplitude of the induced waves to that of the preceding spontaneous activity. The polarity of the sharp peak is shown. The results are plotted on the dorsal surface of the left olfactory bulb. For both stimuli the highest relative amplitudes are found together with negative sharp peaks. The lowest amplitudes correspond roughly to the zone of undeterminable polarity. As for odour specific differences in the response distributions, Fig. 4 shows essentially the same picture as Fig. 2.

The relationship between stimulus concentration and response magnitude at different recording positions is demonstrated in Fig. 5. The figure shows the stimulus/response curves obtained from 4 positions on the olfactory bulb during stimulation with different concentrations of methionine and "crude char odour". Each point represents the mean value of the results from 2-6 recordings.

The responses to methionine show the clearest picture. The stimulus/response curves obtained at different positions were not parallel but radiating from a common origin at 10^{-6} M. The slopes of the curves above this point correspond to the responses at the higher



Dispersion between the amplitude of the induced waves and that of the DC potential shift is seen. Recordings from two positions on the left olfactory bulb of clear starling sturgeon skin at 10^{-8} – 10^{-4} M concentrations. Symbol shapes refer to the recording positions on the sketch. Whole indicates that induced waves are clearly seen.

of mixture of amino acids produced spatially different response patterns, mixtures of parts of the same amino acid solutions in the concentrations given in the figure tested. The response to the mixture of all the 8 amino acid solutions is shown at the top of Fig. 7. The mixtures elicited much larger responses (measured as DC-shift) than any one of their constituents, still without any sign of spatial shift. However the response to any of the mixtures was less than that expected if it had been a sum of independent responses to each of the amino acids at their actual concentrations in the mixtures.

"Fish odour" samples usually showed small but clear differences in their spatial response patterns when tested on the same animal. In the whole material, however the responses were overshadowed by the individual variation of the preparations. Different samples from the same population and therefore presumably the same "fish odour" gave different response distributions as well. The response distribution to a given "odour" could therefore only be coarsely predicted from the responses to other "fish odour" samples and the amino acids. Nor could the response distribution give any safe indication of which population an unknown odour sample came from.

Individual variations

The results often differed from one preparation to another as if the bulbs were slightly moved or rotated in relation to each other. This was most clearly seen when individual

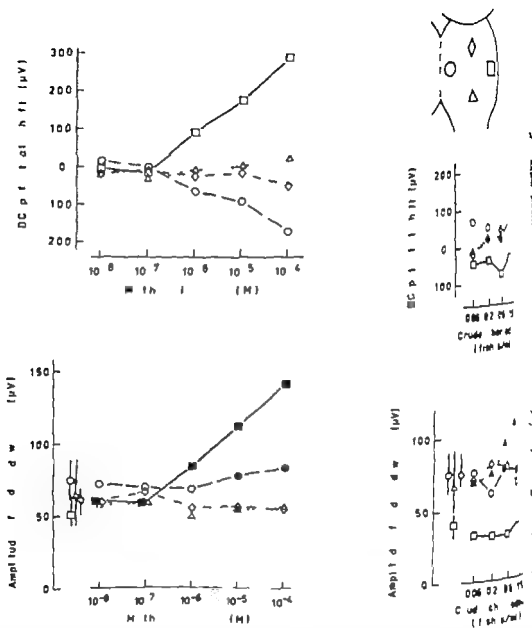


Fig. 5. The magnitude of the DC potential shift (upper two diagrams) and the amplitude of the AC component of the recording (lower diagrams) at 4 positions in the olfactory bulb of a char during stimulation with methionine (right) crude char odour (left) at different concentrations. Graph symbols correspond to the recording positions shown on the sketch in the upper right corner. In each of the two lower diagrams the mean and range of the spontaneous activity at each position is shown to the left. Filled symbols indicate that induced waves were clearly seen. The polarity of the peaks is shown at the right end of the curves, \pm indicating undeterminable polarity.

Similarity between stimuli

Only slight differences were seen between the responses to the eight amino acids used in the present study. No differences in their spatial response distributions were seen. The differences, as demonstrated in Fig. 7 were limited to small variations in the AC to DC amplitude ratio.

potential spread in a volume conductor a weaker positive potential appeared on the so side of the bulb in relation to the negative zone. A prominent positive DC shift was expected in the center of the bulb. This was, however, not found. In the present penetrations no DC shift was evident in the center. It is likely therefore, that a part of C signals reflects events in structures confined to the surface of the bulb, e.g. the olfactory nerve terminals. The question remains if the slow positive DC potential shift observed caudally on the bulb reflects the contribution by postsynaptic elements like the cell dendrites or has an origin entirely different from the rostral negative one.

Induced waves may be of relatively constant amplitude throughout the stimulation, constant amplitude, or appear as spindle-like bursts of waves (Ottoson 1959 a). The resting frequency is species- and temperature-dependent (Dupé and Godet 1969, Gray, Low, Rogers and McLennan 1970, Doving and Beighaug 1977) ranging from about 2 in mammals (Adrian 1950) down to 3 Hz in fish bulbs at a temperature of about 5°C. Baumgarten, Green and Mancía (1962) have made simultaneous surface and depth recordings from the olfactory bulb of the rabbit. From the surface all through the external plexiform layer down to the mitral cell layer the induced waves were exactly in phase but decreasing amplitude. In the mitral cell layer the waves almost vanished, and in the internal plexiform layer the induced waves showed the opposite phase as the surface waves did. The cell waves thus did not behave like a projection of moving dipoles, but like a sum of many oscillating ones. Unit activity was phase locked in such a way that action potentials in the cells of the different layers occurred predominantly at the negative going phase of the induced waves recorded from the same layer. Their results indicated that the induced waves are due to synaptic events between secondary and tertiary neurons.

In the present study the occurrence of negative sharp peaks in the induced waves is closely related with the area giving the highest amplitude in response to the same stimulus. The simultaneous recordings referred to in the present work clearly show that induced waves with positive sharp peaks are just the same signals seen from the reverse, the greater distance from the wave generating layer being responsible for the lower amplitude. The different phase of the induced waves from that of the DC component shows itself in the present results in the difference in their spatial distribution.

Specific spatial distribution of responses

An olfactory stimulus influences the olfactory bulb in such a way that a synchronized oscillating activity is produced in a specific population of secondary neurons. The specific location of activated neurons tells the fish about the odorant to which it is exposed. From an experimenter's point of view the surface potential changes reflect the localization of the synaptic events connected to the specific activity.

In most preparations the results indicate that the bulbary information treatment on amino acids takes place mainly in the lateral and perhaps the ventral parts.

From single cell recordings in the olfactory bulbs of goldfish (Meredith 1974) and rainbow trout (MacLeod 1976) it has been found that the amino acids have unequal, yet overlapping cortical representation in the bulb. On the primary level, cross-adaptation studies on the olfactory epithelium of Atlantic salmon (Søtterhaug and Søtterhaug 1971) showed that

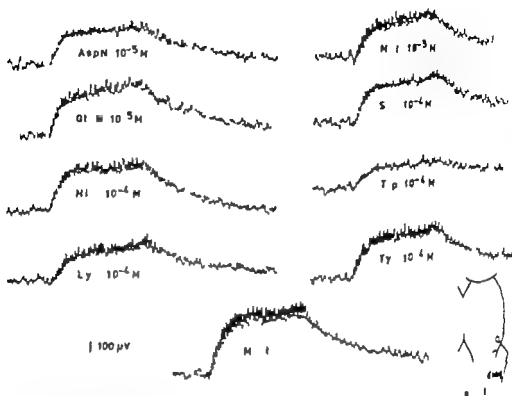


Fig. 7 Comparison of the responses to 8 amino acids with the one to the mixture of even parts of 16 solutions. Note the differences in the AC to DC amplitude ratio for the different amino acids & greater response magnitude of the mixture.

division lines between regions of positive and negative sharp peaks of the induced waves were estimated. The sensitivity of the preparations varied from one individual to another. In general the most sensitive preparations were found during the summer and the least sensitive during the early spring. Minor stimulus specific differences in the sensitivity were also seen. No conclusion on this observation can be drawn however as the experiments were performed too irregularly which may have caused differences in the experimental technique.

Discussion

On the origin of the bulbar potentials

Olfactory bulb responses in fishes show the same general characteristics as those in higher vertebrates. The surface potential changes induced in the olfactory bulb by olfactory stimulation consist of two separable events (Ottoson 1959 b), a sustained or DC potential and the "induced waves" (Adrian 1950).

Ottoson (1959 b) demonstrated that centrifugal stimulation of olfactory tract blocked the induced waves leaving the DC component of the olfactory response intact. He concluded that the results spoke in favour of a presynaptic origin of the slow response, but did not exclude the contribution by postsynaptic elements. This view is supported by the present results. The greatest DC shifts found were constantly negative. As expected according

potential spread in a volume conductor a weaker positive potential appeared on the side of the bulb in relation to the negative zone. A prominent positive DC shift was noted in the center of the bulb. This was, however, not found. In the present penetrations no DC shift was evident in the center. It is likely therefore, that a part of signals reflects events in structures confined to the surface of the bulb, e.g. the olfactory terminals. The question remains if the slow positive DC potential shift occasionally on the bulb reflects the contribution by postsynaptic elements like the cell dendrites or has an origin entirely different from the rostral negative zone.

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In the present study the occurrence of negative sharp peaks in the induced waves is closely related with the area giving the highest amplitude in response to the same stimulus. The time course recordings referred to in the present work clearly show that induced waves with positive sharp peaks are just the same signals seen from the reverse, the greater distance from the wave generating layer being responsible for the lower amplitude. The difference in the induced waves from that of the DC component shows itself in the present results as a difference in their spatial distribution.

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between receptors sensitive to some of the amino acids. In the present study the response to the different amino acids show slight differences in the AC/DC amplitude ratio while they show the same response distribution. The tests with the amino acid mixtures response magnitudes intermediate between that expected if all the amino acids were to act as one and that if they had no receptor site in common. These observations are consistent with both the difference and the similarity in the neuronal representation of these odors.

The rostromedial third of the bulb is presumably the main site in the bulbular level for formation of a response concerning the "fish odours". The area is overlapping with the rostral part of the amino acid field. Skin mucus samples of salmonid fishes contain free amino acids (R. Selset 1975 personal communication) as well as components which may act as pheromones (Nordeng 1971, Døving, Nordeng and Oakley 1974). As an odorant the solution may thus be multipotent, causing the response distribution to be an intermediary between that of the amino acids and that of the pure "fish odour". Stimulus components causing medially located response patterns are not yet identified in odorant samples tested, elicited responses which were too much alike to make the preparation suitable as a bioassay for population specific odorants. It is even not evident that components are unique to the fishes. The term "crude fish odour" has, however been used in this article due to the origin of the actual odorant samples used and in the absence of precise terms.

As to the hypothetical pheromone function of such components it is interesting to see the response distribution they cause. This distribution corresponds well with the distribution in the olfactory bulb which according to the anatomical investigations (Sheldon 1912, Hildebrand 1920) has a direct connection with the magnocellular part of the preoptic nucleus in the hypothalamus. The electrophysiological work by Kandel (1964) indicates that through this connection pituitary activity may be influenced by olfactory stimulation. The olfactory bulb is most likely to produce endocrine changes in the fish should consequently be a "fish odour".

Spatial variation in the response distributions with different stimulus concentrations has not been clearly observed, but is likely to occur with crude mixtures like the "fish odour" components giving different spatial response patterns reach their threshold or saturation concentrations at different mixture concentrations. This may be the explanation for the indistinct results to stimulation with "crude char odour" in Fig. 5. Such effects may also occur using pure substances like amino acids, as each of these may act as an odorant due to different receptor specificity in different parts of the molecule.

The magnitude of the bulb surface response has been used by several authors in the evaluation of olfactory sensitivity to certain odorants in different individuals of salmonid fish. Stimulus/response curves show that this evaluation may be fairly reliable as long as the same recording positions and stimuli are used. The spatial variation in the slope of the curves implies, however, that the sensitivity of the olfactory organ in terms of threshold concentration may be the same in different individuals even if response magnitude at threshold concentration is highly different, particularly near to the dorsal midpole of the bulb which is the most accessible part of it. Individual variations in the orientation of the bulb with regard to the odour specific areas or small variations in the position of the recording electrode may therefore, produce marked changes in the response ampli-

present results confirm that the principle of spatial differentiation of odour specificity is valid for teleosts as well as for air breathing vertebrates. On the basis of the distribution of the primary olfactory neurons in the smelt (*Osmerus eperlanus*), Holmgren stated in 1920 that an olfactory map, "eine Riechkarte" existed on the olfactory bulb in regard to the fish he was half a century ahead of his time.

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Neuroeffector maturity of portal veins from newborn rats, rabbits, cats and guinea pigs

By

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Abstract

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Isolated portal veins from newborn and adult rats, rabbits, cats and guinea pigs are studied as spontaneous activity and induced responses to transmurial nerve stimulation (NS), noradrenaline, acetylcholine and BaCl_2 were quantitated. In agreement with our previous observations the newborn rat was found to lack spontaneous activity and responsiveness to applied stimuli. The newborn rabbit portal vein showed inconsistent and weak responses. In the neonatal cat and portal veins the pattern of the spontaneous activity and induced responses resembled those from adult animals. It is concluded that the portal vein maturity at birth in several respects corresponds to the level of somatic development of the animal. Furthermore, the previously described neuroeffector development of the portal vein during the postnatal period in the rat corresponds to events in cats and guinea pigs which are more highly developed at birth. Consequently the lack of spontaneous activity during the first week of life does not seem to be secondary to the haemodynamic adjustments during the neonatal period. Rather the postnatal development of the portal vein occurs according to a genetically predetermined schedule, possibly in parallel with the development of vascular control in general.

Spontaneous phasic activity is a characteristic functional feature of the longitudinal muscle layer of the adult rat portal vein. In a previous study (Ljung and Stage 1977) we found that coordinated myogenic contractions did not appear in the developing portal vein until the beginning of the third postnatal week. Weak responses to exogenous noradrenaline (NA) and to transmurial nerve stimulation (NS) could first be elicited in the middle and the end of the first week of life, respectively. The sensitivity to these stimuli increased coincidentally to the onset of spontaneous activity during the second postnatal week. The most likely explanation for the delayed onset of responsiveness was considered to be the normal maturation process of smooth muscle and nerve development. However, other possible, but less likely explanations, such as hormonal influences during the lactational period and secondary haemodynamic changes at birth in the splanchnic circulation, must also be considered as well.

In the present experiments, the neuroeffector function of portal veins from newborn and adult rabbits, cats and guinea pigs have been compared. The offspring of these animals at varying degrees of maturity at birth but with presumably similar exposure to maternal influences and comparable hemodynamic alterations at birth.

Methods

Portal vein preparations from newborn (0-7 days of age) and adult animals of 4 different species (rabbit maturity at birth) were studied. Division of the animals by age and species is as follows: guinea pigs—6 newborn (10 g), 4 adults (250 g); rabbits—7 newborns (30 g), 3 adults (1.5 kg); guinea pigs—12 newborn (120 g), 1 mother cat (4.2 kg) and guinea pigs—6 newborns (95 g), 2 mothers (760 g). Each was killed by decapitation, blow to the head, or an overdose of ether. The portal vein, as it exited from the liver, was exposed. In most cases the entire vein was used, but in adult guinea pigs, and cats longitudinal preparations measuring 10-15 mm in length and 2-3 mm in width were used. The vein was mounted under tension in an organ bath containing 40 ml of Krebs solution (composition as in bioassay) and isometric contractile activity was recorded on Grass polygraph (model 7) by an Grass force-displacement transducer (FT 03C). The level of passive tension was determined initially for each preparation by adjustment of its length to that level at which optimal active force was yielded by 10 periods of transmural field stimulation at an exposure rate of 8 Hz. Transmural stimulation from newborn rats and rabbits passive force of 1 and 2 mN, respectively was applied. An incubation period of 60 min was allowed before beginning the experiment.

Experiments are performed according to the following protocol. At the end of the accommodation phase spontaneous activity was quantitated. Then the adrenergic nerve plexus of the portal vein was activated by transmural field stimulation (TFS) during 1 min period by use of Grass stimulator model 84B, each provided square wave impulses of 0.8 ms duration and 15V in amplitude, in platinum electrodes mounted on either side of the preparation, by means of constant voltage. The impulse rate of 32 Hz was chosen to ensure maximum or supramaximal response to transmural stimulation (cf. Ljung *et al.* 1975). Responses were then elicited by 3 min exposure periods to norepinephrine (NA) 10 μ M, tyramine (Tyr) 0.1 mM, acetylcholine (ACh) 1 mM and BaCl_2 5 mM. These concentrations were chosen from preliminary experiments to provide maximum contractile responses to each stimulus. Adequate time was allowed between drug exposures for complete return to baseline activity. At the end of the experiments the length of the mounted tissue was measured by means of a dissecting microscope with micrometer eye piece. The preparations were then removed, blotted between papers and weighed on a Cahn microbalance. The values for weight and length are used to calculate tissue cross-sectional area, assuming density of 1.

Force values for all responses were calculated in absolute terms as maximum force (mN), maximum rate (mN/cm²) and also expressed as percentage of maximum NA responses. Comparison of the means between the two age groups was made by use of Student's *t*-test in groups with adequate numbers for such comparison. A statistically significant difference was considered to exist between the compared groups when the *p* value was less than 0.05.

The standard Krebs solution had the following composition in mM: NaCl 122, KCl 4.7, NaHCO_3 15, CaCl_2 1.8, MgCl_2 1.0, CaCl_2 2.4, glucose 11.5 and $\text{CaNa}_2\text{-veronate}$ 0.026. It was modified for use in experiments by the addition of 30 mM sucrose. The bath solution was bubbled with 4 per cent CO_2 in O_2 to maintain pH of 7.35 at the temperature of 37°C. Drugs used were 1- α -naphthylamine HCl (1- α -naphthylamine), Co. St. Louis, Mo. (U.S.A.), tyramine chloride, acetylcholine chloride and BaCl_2 (E. Merck, Darmstadt, Ger.) Freshly prepared, saline-diluted drugs in volumes of 0.4 ml were injected directly into 40 ml bath to give the desired concentration.

Results

Activity at birth. The great variability in the maturity level of the four different animal species at birth was readily apparent by examination of their physical appearance. At the minimum maturity level on the developmental scale was the furless, sightless and immobile

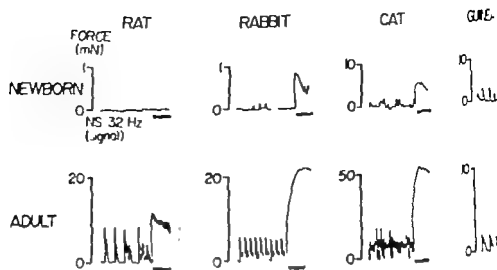


Fig. 1 Experiment I recordings of spontaneous activity and responses to transmural field stimulation (supramaximum impulse rate 32 Hz). Note lack of responsiveness in neonatal rat and postnatal neonatal rabbit portal vein.

newborn rat which is born after a gestational period of 21 days and is totally dependent on the mother for survival. At the other end of the scale was the fur covered, open-mouthed newborn guinea pig, which is born following a gestational period of 68 days. The furless and sightless newborn rabbit with a gestational period of 31 days and the fur covered newborn cat with a gestational period of 63 days fit in between the guinea pig on this developmental scale. Thus, placement of the four species in ascending maturity at birth is as follows: rat, rabbit, cat and guinea pig.

Spontaneous activity and responses to nerve stimulation. The experimental results are illustrated in Fig. 1. Figure 1 illustrates species variation in onset, pattern and magnitude of spontaneous activity and response to nerve stimulation at a frequency of 32 Hz in newborn as compared with adult portal vein preparations. Complete lack of activity is noted in the portal vein of the newborn rat. The portal vein of the newborn rabbit sometimes exhibited a few spontaneous phasic activity occurring irregularly at the beginning of the experiment and in most cases a clear cut but weak response to nerve stimulation at 32 Hz was obtained. All preparations from the newborn cats and guinea pigs manifested spontaneous phasic activity and neurogenic responses. Although the pattern of spontaneous contractions varied, forceful spontaneous contractile activity appeared throughout the experiments. Likewise the magnitude of response to maximal NS was greater than in the rat and rabbit.

All adult preparations exhibited spontaneous activity and NS response. As seen from Fig. 1 the frequency and magnitude of spontaneous contractions were greater in the adult portal vein than in the vessels from newborn animals. It is not possible to quantitatively compare contraction amplitudes or degree of synchronization by the tracings of Fig. 1. Longitudinal strips of adult portal veins from the rabbit, cat and guinea pig were used

conference of the vessel was included in all preparations from newborn animals : adult rat.

NA. The relationships between the number of tissues responding to exogenous NA (10 μ M) and the total number studied and the peak force response magnitude Table I. In 4 out of 6 portal veins from rats studied during the first week of life 7 rabbit portal veins no NA responses could be elicited. In all other preparations one from newborn kittens and guinea pigs clearcut responses were obtained.

Amplitude of spontaneous activity and responses to NS tyramine, acetylcholine and Table 1 the number of portal vein preparations which exhibited spontaneous responded to transneral nerve stimulation (32 Hz), to tyramine (0.1 mM) to ACh (1 mM) and to BaCl₂ (5 mM), respectively has been expressed as a fraction of number of tissues studied. In some experiments the whole sequence of non-stimulations was not applied, hence the variation in number of observations. In where a NA response was obtained the peak force of spontaneous activity and the onset amplitudes were expressed as a percentage of the maximum NA response. Significant differences between such relative response values of portal veins from newborn animals were found for spontaneous activity nerve stimulation and in the rat and for nerve stimulation in the rabbit. It is of particular interest that the responses of newborn cats and guinea pigs corresponded to those of adult rats.

Discussion

Portal vein did not respond to nerve stimulation during the first week of life and to exogenous NA were only obtained in those rat preparations which were studied at the end of the first week of life. These findings are in accordance with our previous findings (Lundberg and Sjöberg 1975) where the ontogenetic development was examined in several vein preparations from each day during the first 6 postnatal weeks. It was found that onset of responses to NA, to ACh and to nerve stimulation was 4, 4 and 6 days, respectively. Furthermore, phasic spontaneous activity occurred rather abruptly at the 16th day. This functional development is paralleled by morphological differentiation and in-growth of terminal adrenergic nerve fibers (Lundberg et al. 1976). It is thus that the neuroeffector maturation of the portal vein occurs postnatally in the rat. In animals, notably the guinea pig and the cat, spontaneous myogenic activity of the vein and responsiveness to external stimuli developed earlier than in the rat. It is difficult to study a limited number of portal vein preparations from these newborn animals since we found that they do exhibit spontaneous activity and do respond to adrenergic stimuli during the first few days of life. Moreover we only included a few adult animals since their portal veins have previously been studied in these respects (for review see Lundberg 1970). The rather limited number of observations precludes any detailed quantitative comparisons but it seems possible to conclude that in the cat and the guinea pig the neuroeffector function of the portal vein is completely developed in all respects at birth since the relative magnitude of spontaneous activity and responsiveness to a variety of stimuli acting at different levels in the neuroeffector chain are comparable.

TABLE I (A) Responses to exogenous noradrenaline of portal veins from various species and animals expressed as relative number of tissues responding to the point and up (mN) and peak tension (mN/mm²) of responding preparations. Mean \pm S.E. Note the portion of the media circumference was included in the preparations of portal veins in rabbits, cats and guinea pigs. Statistically significant differences ($p < 0.05$) between animals

	Rat		Rabbit	
	Newborn	Adult	Newborn	Adult
A				
Noradrenaline 10 μ M				
No responding/No tested	2/6	4/4	5/7	3/3
Max force (mN)	0.11 \pm 0.01	10 \pm 0.9	0.54 \pm 0.3	1.1
Max tension (mN/mm ²)	3.3 \pm 3	2. \pm 3	1 \pm 1	1.1
B				
No responding/No tested max force (max NA response)				
Spontaneous activity	0/6	0	4/7	18 \pm 8
Nerve stimulation (32 Hz)	0/6	0*	4/4	96 \pm 2
Tyramine 0.1 mM	0/6	0	4/4	75 \pm 9
Acetylcholine 1 mM	3/6	114 \pm 14	2/2	105 \pm 5
BaCl ₂ 5 mM	3/6	10. \pm 2	4/4	109 \pm 3
			3/5	90 \pm 5
			3/5	85 \pm 5

(Table I) (Gerova *et al* 1974). Naturally maturation of the smooth muscle may that increased tension responses, expressed as force per smooth muscle cross section are obtained with advancing age (see Ljung and Stage 1975). Indeed, the tension (mN/mm²) given in Table I indicate that increase in contractility does occur during development in all species studied except the guinea pig. However the cross section was calculated from values for weight and length in our experiments and thus the value of the actual effector tissue, i.e. the smooth muscle of the longitudinal media layer, has been determined directly. The relative amount of longitudinal smooth muscle in the wall of the portal vein can be expected to vary during development (cf Lundberg *et al*). Consequently a definite conclusion regarding changes in vascular smooth muscle contractility cannot be drawn from the present results alone.

In the newborn rabbit portal vein weak spontaneous activity was found in 4 preparations. Furthermore, adrenergic responses were present in the majority of the greater responsiveness seems to occur in the newborn rabbit portal vein than in the rat.

Altogether the present results strongly indicate that the degree of portal vein maturation at birth is related to length of gestational period and stage of development of the foetus studied. Such variation in vascular development with gestational length is in agreement with a recent *in vivo* study on the carotid artery of the fetal lamb (Su *et al* 1977) where evidence of functioning neuroeffector control was demonstrated already at the beginning of the second trimester. Consequently the dramatic changes which occur in the portal vein of the rat during the first few postnatal weeks seem to take place during fetal development in animals which are more highly developed at birth such as cat, guinea pig and sheep. In the rabbit portal vein assumes an intermediate position. This seems to rule out the stages of postnatal development previously observed in the rat portal vein are also

An electronic differential pressure flowmeter and a resistance meter for continuous measurement of vascular resistance

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Abstract

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An electronic differential pressure flowmeter for mean blood flow measuring and its use as a resistance meter for continuous recording of vascular resistance is described. The flowmeter allows a linear registration also of the small flow range (< 10 ml/min), thus appropriate for use in experimental animals.

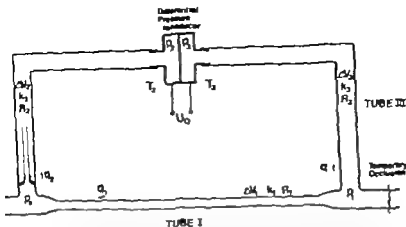
Keywords. Blood flowmeter vascular resistance meter differential pressure flowmeter

One of the most important variables in the peripheral circulation is vascular resistance. It is usually determined by manual calculation of the ratio of mean perfused pressure and mean blood flow. This is, however, a time-consuming process which for practical reasons only gives scattered information. Continuous recordings of vascular resistance could be readily obtained by feeding the output signals from a differential pressure transducer and a flowmeter into an electronic divider circuit. Instruments with high accuracy and stability must be used, however, to obtain reliable continuous measurements of resistance.

A problem in this context is encountered in studies on small experimental and minute tissue blood flows in the range below 10-15 ml/min, since most commercial flowmeters, like the electromagnetic one, show poor technical characteristics in this range. This paper describes an extracorporeal differential pressure flowmeter for measurement of minute blood flows and its use for continuous recordings of total and peripheral resistances in the vascular bed of skeletal muscle in the cat.

The differential pressure flowmeter

Theoretical considerations The flowmeter device consists of an extracorporeal branching rigid tube with uniform cross-sectional area across the differential pressure measurement points.



Schematic illustration of the differential pressure flowmeter under temporary occlusion of flow after consists of tubes I, II and III. Here ΔV —reduced volume change, R —tube resistance, n, T —time constant and k —tube elasticity modulus. Note the special construction to obtain equality by making the relationship $R_9 = R + R_8$ and $k_4 = k_3$ (see text).

only recorded. A mathematical analysis of the pressure–pulsatile flow relationship between fluid in such a system was made by Fry, Mallory and Casper (1956) by solution of Navier–Stokes equations

$$p(t) = \rho \Delta x \frac{du}{dt} + a u(t),$$

$p(t)$ is the pressure difference, as a function of time, over the distance (Δx) between measuring sites, $u(t)$ blood velocity as a function of time, ρ fluid density and a a constant relating the frictional pressure drop. The velocity profile is assumed flat. When measuring mean flow in the present system, the term $\rho \Delta x \frac{du}{dt}$ can be ignored since it is negligibly small compared to $a u(t)$, (cf McDonald, 1974). Thus

$$p(t) = a u(t),$$

with constant tube cross-sectional area

$$p(t) = b q(t)$$

where b is a constant and $q(t)$ volume flow as a function of time. This equation is approximately valid also for a non-Newtonian fluid, like blood for the tube dimensions and flow rates used in the present device (see below).

The basic principle of the flowmeter is shown schematically in Fig. 1. It consists of a tube (I) diverting blood flow (q_1) from the proximal to the distal end of a divided tube. The pressure drop over tube I is measured with a differential pressure transducer (usually no membrane displacement (National Semiconductor Corp., LX1601D)) between tubes II and III, the former supplied with an inner tubing of smaller size for a special purpose (see below). It is essential that the pulsatile pressure at the entrance of tube II is admitted to either side of the differential pressure transducer without significant phase shift. This implies that the time constant (T_2) of tube II should be identical to that (T_1) of

tubes I + III which can be accomplished by choosing appropriate values for the flow resistances, R , R_1 and R and for their elasticity moduli, k_1 , k_2 and k_3 (see Fig. 1) according to the calculation below. Since the time constants are independent of flow, the calculation refers to the zero mean flow situation accomplished by temporary occlusion of the distal artery (Fig. 1). Pulse pressure will then cause minute flows, q_1 , q_2 and q_3 , in the three limbs of the flowmeter. p_0 , p_1 , p_2 and p_3 denote pressures at the sites indicated in Fig. 1. ΔV_1 , ΔV_2 and ΔV_3 the pressure induced volume changes of tubes I, II and III and k_1 , k_2 and k_3 their elasticity moduli. Then $dp_1 = -k_1 dV_1/dt = -q_1$, $dp_2 = -k_2 dV_2/dt = -q_2$, $dp_3 = -k_3 dV_3/dt = -q_3$.

The relation between flow, resistance, and pressure drop for the three tubes is expressed

$$q_1 R = p_0 - p_1, \quad q_2 R_1 = p_0 - p_2, \quad q_3 R_2 = p_1 - p_2$$

If $1/k_1 \approx 0$ (i.e. tube I is made rigid), the equations above give, after Laplace transformation, the following solution

$$p_1 = p_0 \frac{1}{1 + \frac{R_2}{k_2} s} = p_0 \frac{1}{1 + T_2 s}$$

$$p_2 = p_0 \frac{1}{1 + \left(\frac{R_2}{k_2} + \frac{R_1}{k_1} \right) s} = p_0 \frac{1}{1 + T s}$$

why

$$T_2 = \frac{R_2}{k_2}, \quad T = \frac{R_2 + R_1}{k_2}$$

Thus, if k_1 and k_2 are made identical, the relation $T = T_2$ is valid when $R_2 = R_1$.

Design of flowmeter. The flowmeter was designed to fulfil the criteria mentioned above. The $k_1 = k_2$ relation was accomplished by making tube II (outer) and tube III of equal length and of the same semirigid polyethylene material. The $R_1 = R_2 = R$ relation was accomplished by the addition of the small tube inside tube II with a resistance identical to R . It is obvious that the membrane of the differential pressure transducer must operate without significant displacement, a criterion fulfilled by the chosen piezoresistive transducer (National Semiconductor, LX1601D). Tubes II and III are filled with fluid without air trapping. To avoid deterioration of the pressure transducer its internal space was filled with the completely inert FC40 (3M Brand Inert fluorochemical liquids, Chemical Division 3M Company). With this design there was virtually no phase lag between p_1 and p_2 (Fig. 1), as evidenced by the absence of arterial pulse pressure oscillations at zero flow during distal artery occlusion. The transducer was hermetically encapsulated and thermoisolated to avoid damage by contact with electrolytes and interference by external temperature variation.

The magnitude of the resistance in tube I (R_1) (and hence, also R_2) can be varied within a wide range. The value chosen depends on the range within which blood flow is to be measured, on the upper limit of the arterial blood pressure fall across tube I which can be accepted, and on the sensitivity and accuracy of the differential pressure transducer.

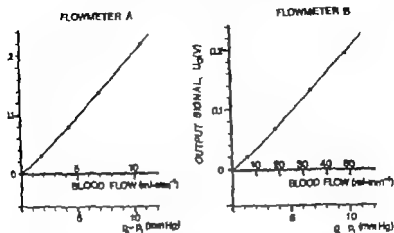


Figure 1. Calibration curves of the relation between blood flow and the output signal (U_O) from the transducer for two different flowmeter resistances. Flowmeter pressure falls ($p_a - p_v$) at different Q illustrated as second abscissa. Flowmeter A represents tube of 85 mm in length and 1.1 mm internal diameter while flowmeter B represents a tube of 85 mm in length and 1.75 mm in internal di-

ameter as small as possible to limit the arterial pressure fall across the flowmeter. With present design of the chosen differential transducer flows within the range from 0 to 10 ml/min can be reliably recorded for an arterial pressure fall across the flowmeter to 10 mm Hg.

Figure 1, panel A and B, shows calibration curves for two flowmeter types, one with relatively large R designed for measurements in the low flow range (length of tube 1.85 mm, internal diameter 1.1 mm) and another with smaller R for larger flows (length of tube 1.85 mm, internal diameter 1.75 mm). Blood flow (for calibration purpose measured with a graduated cylinder) is plotted versus the output signal (U_O) from the differential transducer. Along the x-axis is also plotted the pressure difference across tube 1 ($p_a - p_v$). As can be seen, linearity is satisfactory. This fact also indicates that viscosity changes of the blood within these flow ranges of velocity were insignificant. The present flowmeter design gives a baseline drift of less than 2% of full scale deflection corresponding to a maximal pressure fall of 10 mm Hg across the flowmeter at standardized conditions with constant ambient transducer temperature and constant blood viscosity. Flow calibration including zero flow calibration is repeated to be tested once every hour.

The vascular resistance meter

Vascular resistance is determined by the ratio of mean perfusion pressure and mean blood flow. Total vascular resistance in a peripheral vascular bed is thus measured as: (mean inflow pressure - mean venous outflow pressure) / mean blood flow. Mean perfusion pressure can be readily obtained from the filtered output signal of a differential pressure transducer and mean blood flow from the filtered output signal of an arterial flowmeter.

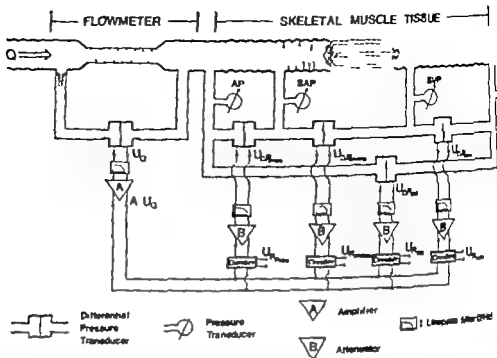
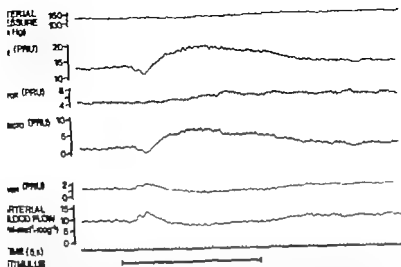


Fig. 3 Illustrates a principle scheme for vascular flow resistance meter when used on skeletal recording total resistance (U_{total}), proximal arterial resistance (U_{prox}), microvascular resistance (U_{micro}) and large venous resistance (U_{large}). The scheme also includes continuous registration of flow and tissue pressure fall as $B \cdot U_{\Delta P}$ as well as arterial pressure (AP), small artery pressure (SAP), small vein pressure (SVP) and venous pressure (VP).

Continuous measurements of vascular resistance can be obtained by feeding these two into an electronic analog divider.

In the present resistance meter mean perfusion pressure was measured with a N. Semic. LX1601D differential pressure transducer and mean blood flow with the meter described in the foregoing, while vascular resistance was obtained with the Analog Devices 532 KD divider. Since the voltage ratio of the two input signals to the electronic divider circuit must never exceed one, the present system requires appropriate attenuation of the pressure signal and/or amplification of the flow signal.

Fig. 3 illustrates schematically how the flowmeter and the resistance meter can be used for the measurements of total and segmental resistances in the vascular bed of the skeletal muscles in the cat. The flowmeter is inserted in the cognate artery for continuous recording of arterial blood flow. Arterial inflow pressure (AP), small artery pressure (SAP), small vein pressure (SVP) and venous outflow pressure (VP) are monitored from indwelling catheters as described elsewhere (Grände, Lundvall and Mellander 1977, Grände and Mellander 1977) and recorded by Statham transducers. The differential pressure transducers provide signals for the pressure drops (ΔP) across the whole vascular bed (AP-VP), the proximal arterial vessels (AP-SAP), across the microvessels (SAP-SVP), and across the large venous vessels (SVP-VP). The blood flow signal and the differential pressure signals are fed into the analog dividers to provide continuous recordings of vascular resistance in the whole vascular bed (R_{total}), in the proximal arterial vessels (R_{prox}), in the microvessels (R_{micro}) and in the large venous vessels (R_{large}).



Polygraph tracings (Gram Polygraph) of effects on arterial pressure, arterial blood flow total (TPR), proximal resistance (R_{prox}), microvascular resistance (R_m) and large venous resistance (R_v) infusion of norepinephrine ($1.0 \mu\text{g} \text{ min}^{-1} \text{ kg}^{-1}$) on sympathetized lower leg skeletal (weight 30 g) of cat, with the experimental approach illustrated in Fig. 3.

resistance (R_m), and in the large venous vessels (R_v) as shown in Fig. 3. Fig. 4 shows how these different vascular resistances are influenced by an infusion of norepinephrine.

Calibration of the flowmeter is performed as described above. Calibration of the total segmental pressure drops is made with the aid of the Statham transducers. Strict zeroing of all differential pressure transducers at zero differential pressure must be made.

Comments

Differential pressure principle for blood flow measurement has been applied in several previous flowmeter designs (e.g. de Burgh Daly 1926, Lawson and Holt 1939, Ueno and Nakai 1955). The very small mean pressure gradient over the flowmeter created during flow measurements has been very difficult to record exactly with the so far described devices (e.g. mechanical, optical, or strain gauge manometers). There have also been other problems with a linearity phase lag of pulse pressure etc. in the previous instruments. For these reasons the method has become of little practical use in biological research. Recent developments in the field of piezoresistive pressure transducers, especially the thin-film IC type (National Semiconductor Corp.), offers the possibility of precise and reliable measurements of minute differential pressures. The present flowmeter in which a thin-film transducer was used and in which the problems with pressure phase lag were largely overcome, seems to provide a means for reliable and linear recordings of mean blood flow, even in the small flow range. The flowmeter can be designed as a small compact unit, is relatively inexpensive, its baseline drift is small and its operation largely independent of

of accidental movements. Since the flowmeter is of the cannulating type, anaesthesia must, however, be administered. There is, further, an inherent pressure drop in a flowmeter in relation to flow which therefore interferes with arterial pressure measurements. This disadvantage can be minimized by choosing a small fixed resistance of the flow (Fig. 2).

The flowmeter has proved to be very suitable as a component in the described recorder which, in turn, offers the possibility of detailed and reliable continuous recordings in various vascular beds of the peripheral circulation.

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Characteristics of static and dynamic regulatory mechanisms in myogenic microvascular control

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Abstract

GRÜNDE, P.-O. and MELLANDER, S. Characteristics of static and dynamic regulatory mechanisms in myogenic microvascular control. *Acta physiol. scand.* 1978. 102: 231-245.

Described static and dynamic components in myogenic microvascular control (Gründe, Lundin 1977) were analysed in this study with regard to their stimulus-effector characteristics. Arteriole resistance responses in the sympathetomized vascular bed of skeletal muscle during graded changes of vascular transmural pressure (P) applied at different rates (dP/dt) from -7.5 to $+7.5$ mmHg/s. The dynamic microvascular resistance responses, developing basis of changing P , were pronounced and distinctly graded in relation to the magnitude of stretch, both with regard to amplitude of resistance response and rate of resistance change per P -increase (dR/dP). The static responses, revealed in the steady state phase of constant increased P , were relatively small and graded in relation to the amplitude of the P increase. Rate-sensitivity in the myogenic control was bi-directional, eliciting constrictory effects (constriction) in response to inhibitory effects (distension) in response to positive values of dP/dt . The dynamic constrictor given dP/dt stimulus increased with increasing amplitude of P and, thereby, increased during dynamic stretches. This effect might be explained by successive activation of myogenic receptor different thresholds. The described rate-sensitivity in the myogenic control system seems to insure stability and sensitivity and thereby can contribute efficiently to well-adapted and re-vascular adjustments.

Myogenic control, local vascular control, myogenic receptor-effector characteristics, rate-transmural pressure change, muscle microcirculation, autoregulation, servo-control

Vascular reactivity has in the past been discussed almost exclusively in terms of constrictor/dilator responses which develop upon a given steady state increase in vascular transmural pressure. Such steady state effects can be considered to be of a static myogenic stimulus related to the amplitude of the transmural pressure. Recent *in vivo* and *in vitro* studies have demonstrated, however, the existence of an additional and highly effective dynamic component in the myogenic vascular control. Most of this was evidenced by *in vitro* observations of electrical and mechanical activity in single-unit vascular smooth muscle when exposed to a given passive stretch or length applied at various rates of length changes, dL/dt (Johansson and Mellander

1975) A strong static stimulus (steady state increase of length by 40%) was shown to elicit only moderate excitatory effects in terms of a 10–15% increase in spike discharge and attendant increase in active force. The dynamic stretch stimulus, operating during the period of increasing muscle length, was much more effective causing graded excitation which at high dL/dt values were up to 20 times greater than those evoked by the static stimulus. Passive shortening at different rates caused graded inhibitory responses below control level, complete inhibition of electrical and mechanical activity being obtained at high negative test values of dL/dt . Further analysis suggested that the dynamic response was more closely correlated to the rate of change of passive tension (dL/dt) than to dL/dt (Sigurdsson, Johansson and Mellander 1977).

Circumstantial evidence for the presence of a dynamic rate-sensitive component in myogenic control *in vivo* was derived from observations of increased vascular tone upon changes of pressure or shift from steady to artificial graded sinusoidal pressure perfusion in the kidney (Basar and Weiss 1969) and upon shift from steady to normal pulsatile perfusion in skeletal muscle (Mellander and Arvidsson 1974). More direct evidence for this concept was recently obtained in a study of total and segmental vascular resistance response in skeletal muscle to standardized transmural pressure stimuli (Grände, Lundvall and Mellander 1977). These stimuli were induced by exposing all parts of the vascular bed to a change of vascular transmural pressure applied at two distinctly different rates, one high and one low. The resistance vessels showed clearcut active constriction upon transient pressure increase and active dilation upon pressure decrease. These responses were especially pronounced in the microvessels where a distinct two-component effector response was obtained. The initial component, developing during the period of changing pressure, was much larger when evoked by high than low rate of transmural pressure change, thus indicating that the response was rate-dependent. The subsequent steady state component revealed during the period of constant increased pressure, was small and rate-independent.

These data indicate that the vascular bed of skeletal muscle is responsive to static as well as dynamic myogenic stimuli. The dynamic responses were almost exclusively confined to the microvessels, though reflected also in the total vascular resistance of the region, while the proximal arterial vessels seemed to be dominated in their control by static myogenic stimuli. The large venous vessels appeared to lack myogenic reactivity.

Little is known about the mode of operation of the myogenic vascular control system or its cellular mechanisms. Apparently the system is more complex than previously believed in view of its recently described rate sensitivity. Further progress in this field requires detailed information about the myogenic stimulus-receptor-effector characteristics. In the present study an attempt was made to define some of these characteristics with regard to the static and dynamic components in myogenic microvascular control.

Methods

Seven cats, b.wt. 2.8–3.6 kg, were used in the study. They were anesthetized with chloralose (50 mg/kg) and urethane (100 mg/kg b.wt.) after induction with ether. Body temperature was kept at $38 \pm 0.5^\circ\text{C}$.

The observations were made on the acutely sympathectomized vascular bed of the lower leg muscle preparation and the experimental set up were basically the same as described previously.

and McSwander 1977). In brief, arterial blood flow to the region was recorded continuously after catheter (750 μ L/kg \pm t.) with pressure gradient flowmeter (Orlidge and Bergström 1978) in a short circuit placed between the femoral and the popliteal artery. This flowmeter device is accurate registration of mean arterial blood flow. Venous outflow of blood from the region was done by the jugular vein catheter inserted in the popliteal vein. Mean arterial inflow pressure (AP), artery pressure (SAP), small vein pressure (SVP) and venous outflow pressure (VP) in the muscle were monitored continuously with the use of Statham transducers (for technical details see Haddy 1974, Orlidge *et al.* 1977). This experimental approach, combined with plethysmographic recordings and transcapillary fluid movements and capacitance responses, can permit reliable estimations of vascular resistance in the region and of segmental resistances in the "proximal arterial vessels", "microvessels" and the "large venous vessels" according to the definitions given in our previous work (Orlidge *et al.* 1977). In the present study only total and microvascular resistances were studied, but the technique was here refined so as to provide continuous resistance recordings with the use of pressure transducers and electronic divider circuits (Orlidge and Bergström 1978). Total or resistance in the muscle region as represented by $(AP - VP)/\text{arterial flow}$ and "microvascular" resistance by $(SAP - SVP)/\text{arterial flow}$ in these recordings, neglecting the effects of reduced passive vascular flow and net transcapillary fluid movement on segmental blood flow. Since such effects hardly affect blood flow in the precapillary vessels, which are the myogenically reactive ones and here the resistance vessels (see Ruzicka), the inherent error in the total and "microvascular" resistance determinations is small and, if anything, will lead to an underestimation of the described active dynamic response to these vessels (see further Discussion).

At surgery and vascular cannulations were completed, the isolated muscle preparation was placed in potassium-controlled plethysmograph (38°C) filled with Tyrode's solution. The entrance of the plethysmograph, through which the arterial and venous cannulas passed, was closed hermetically with the aid of an silicone rubber (Medical Elastomer, Dow Corning). The fluid in the plethysmograph was in connection with an open reservoir placed outside the plethysmograph (Orlidge, Järnålt and Haddy 1974). Recorded hydrostatic fluid pressure in the plethysmograph could be adjusted to desired level by varying the height of the reservoir in relation to the muscle preparation. Fluid displacements between the plethysmograph and the reservoir (recorded gravimetrically) represent changes of tissue volume. At control state, plethysmograph pressure was set at the atmospheric level and VP was adjusted to a level of 75 mmHg creating arterial-transcapillary fluid equilibrium. By lowering the reservoir the plethysmograph pressure, and hence tissue pressure, could be decreased. In this way all parts of the muscle vascular bed could be exposed to graded increases in vascular transmural pressure (P_T) of different amplitudes, and pressure changes were applied at different rates (dP_T/dt) over wide range. Levels of the reservoir to control level permitted decline of vascular transmural pressure at the same graded rates. All measured signals were recorded on Grass polygraph. Resistance recordings were stored on magnetic tape using Instronimation Recorder 100 for later replay on Rika Desk II recorder (to avoid carrier amplifier station). Spread of data is given as S.E.

Results

Animals rested for at least 30 min after completion of the experimental preparation to the recovery from the surgical trauma. Blood flow, the different pressures and vascular resistances in the acutely sympathectomized skeletal muscle region were followed continuously throughout the exp. during which the whole vascular bed was exposed, at intervals, a change of transmural pressure (P_T), accomplished by alteration of extravascular pressure (see Methods). In one series of exps., P_T was always increased by a total of 30 mmHg above the normal control level, but this pressure change was applied at different rates in the dP_T/dt range from 0.32 mmHg/s to 7.5 mmHg/s. In another series, the amplitude of the P_T increase was varied as well. In each observation period, P_T was first increased to the high level at given rate, then maintained constant at this plateau for about 2 min, and finally returned to the control level at the same given rate (see Fig. 1 upper panel). In this way

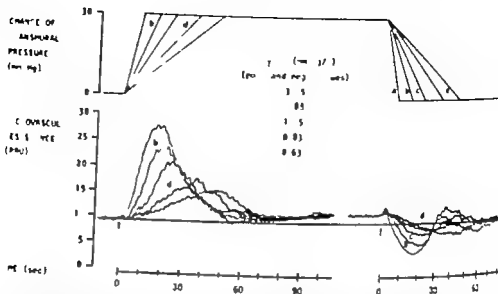


Fig. 1 Myogenic microvascular resistance responses in sympathectomized skeletal muscle in the cat to increase and subsequent decrease in vascular transmural pressure, P_T , by 30 mmHg applied at 5 different rates, dP_T/dt , from 0.63 to 7.5 mmHg/s. The original consecutive resistance recordings are superimposed on top of each other. Note the pronounced dynamic constrictor and dilator responses during the pressure change dP_T/dt , graded in relation to dP_T/dt both for positive and negative values, and the conspicuous small static constrictor response in the steady state period of constant increased P_T .

the resistance responses to graded dynamic transmural pressure stimuli, both positive and negative values of dP_T/dt could be studied ("dynamic" response) as well as the reaction to constant increased P_T ("static" response). These effects were compared with the resistance values in the control period preceding each experimental intervention. The interval between consecutive tests of this type was long enough to permit recovery of vascular reactivity. In some experiments, the vascular bed was first exposed to the highest rate of pressure change and then to gradually lower ones; in the others the sequence was reversed, but the order did not influence the results.

Although the experimental approach used (see Methods) permits detailed observations of total and segmental vascular resistances in the muscle, emphasis will here be placed on the responses of the "microvessels" since, as mentioned, these are the ones which exhibit direct reactivity to dynamic myogenic stimuli (Grände *et al.* 1977). Microvascular resistance as recorded in this study comprises that of small arterial, capillary and small venous vessels (see further below).

Fig. 1 illustrates, in original tracings from one representative experiment, how microvascular resistance in skeletal muscle was influenced by a given total increase in P_T of 30 mmHg applied at 5 different rates (dP_T/dt 0.63–7.5 mmHg/s) and by subsequent decrease in P_T back to the control level at the same given rates. To permit direct comparison of results and to limit space, the consecutive original recordings have been reproduced on top of each other in such a way that the starting points for increase and decrease in P_T respectively coincided on the time scale (see arrows below the tracings and graphical illustration of induced P_T change). Control microvascular resistance before pressure elevation averaged 9.2 ± 0.3 PRU (mmHg ml⁻¹ min⁻¹ 100 g tissue⁻¹) in these 5 observations.

μ of P in each test led to the following general pattern of microvascular resistance (Fig. 1). Within a few seconds after the onset of the P_T rise, resistance started to and in an almost linear fashion reached a peak or transient plateau shortly after attained the final constant level of 30 mmHg above the control value. Resistance declined again, approximately in an exponential manner in spite of the fact that P_T remained constant at the high level. Some 40–60 s after the peak, resistance stabilized at a steady state level averaging only 1.8 ± 0.2 resistance units (PRU) above the initial control value. When P was decreased microvascular resistance, after a small and transient (passive) decline to a value clearly below the control level. The subsequent recovery of microvascular resistance was characterized by an overshoot and/or an oscillatory behaviour before it stabilized at its initial control value. The described changes of microvascular resistance were caused by simultaneous alterations of SAP, SVP and blood flow according to the same general pattern of response as described in detail in our previous publication (see *et al.* 1977).

The resistance data in Fig. 1 demonstrate the existence of a clearcut two-component constrictor response in the microvessels to increased P_T . The initial resistance component, developing during the phase of increasing pressure and, hence, in all probability related to the dP_T/dt stimulus, was large and transient in nature, while the second component remained, though maintained throughout the following period of constant increased pressure. Decrease of P back to the control level caused clearcut active dilator responses during the phase of declining pressure (negative dP_T/dt) before resistance stabilized at the control level. The magnitude of the second component was related to the amplitude of the P_T increase over a wide range (see Fig. 4).

The constrictor and dilator responses were completely absent in the papaverine-treated, fully dilated vascular bed and here reversed to passive vascular distension effects in response to increased, and passive vascular collapse effects, to decreased transmural pressure as described previously (Orlidge *et al.* 1977).

The responses in the normal vascular bed, as will be discussed below, can be considered myogenic in nature. The results, which confirm and extend our previous observations (Orlidge *et al.* 1977) strongly indicate the presence of a rate-sensitive (dynamic) and a static myogenic mechanism in the myogenic microvascular control. Evidence for this interpretation is discussed at length in our previous publication. The rate-sensitive mechanism, in action during the phase of the P change, was bi-directional causing excitatory effects (constriction) in response to positive values, and inhibitory effects (dilation) in response to negative values, of dP_T/dt (or rather some receptor modality indirectly related to dP_T/dt). The static myogenic mechanism, revealed during the steady state of constant increased P , was quantitatively much less effective in evoking microvascular constriction.

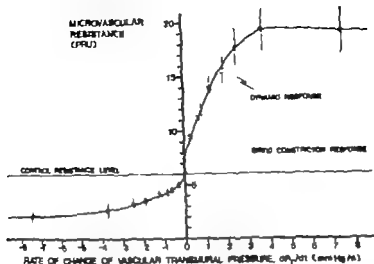
The results in Fig. 1 demonstrate, in particular, that the magnitudes of the dynamic constrictor and dilator responses were distinctly graded in relation to the magnitude of dP_T/dt over the range analysed. When P was increased at the rate of 3.75 mmHg/s, microvascular resistance thus increased by 18.5 resistance units (PRU) above control; the corresponding rate at the low rate of 0.63 mmHg/s was only 7 units. The true dynamic resistance effects must be 1.8 units less than these values, if the whole static constrictor response (1.8 PRU)

can be considered to be fully developed already at the initial peaks. When P_T was decreased at the high (3.75 mmHg/s) and low (0.63 mmHg/s) rates, resistance declined by 6 and 1 units, respectively below the initial control level (thus representing a true dynamic response). The dynamic constrictor and dilator responses at the intermediate dP_T/dt values (Fig. 1) ranged between the mentioned extremes. Note also that there was a distinct delay between the rapidity of the resistance response (both with regard to onset and decay) and the dP_T/dt value. The resistance response thus started 1–2 s after the commencement of the P_T change at the highest test value of dP_T/dt but was somewhat more delayed at lower rates (Fig. 1). The resistance change per unit time, dR_{micro}/dt , was graded in relation to the dP_T/dt values, both positive and negative (see further below Fig. 3).

The described dynamic resistance responses in the microvessels were reflected qualitatively and in rough terms also quantitatively in the simultaneously obtained recordings of vascular resistance in the muscle region. This finding warrants our previous conclusion (Grände *et al.* 1977) that dynamic reactivity is mainly confined to the microvessels. The steady state constrictor response in the whole vascular bed to constant increased P_T exceeded, however, that in the microvessels, a result in agreement with our previous conclusion that the proximal arterial vessels, like the microvessels, exhibit clearcut reactivity to myogenic stimuli.

Seven series of observations of the type illustrated in Fig. 1 were performed. Fig. 2 summarizes the results in terms of the average dynamic microvascular constrictor and dilator responses to dP_T/dt stimuli over the entire range investigated (from +7.5 mmHg/s to -7.5 mmHg/s). The average static constrictor response to the standardized increase of P_T by 30 mmHg is also illustrated. It can be seen that the microvessels exhibited smoothly graded dynamic constrictor responses to excitatory dP_T/dt stimuli (pressure increase) in the entire range of test with a maximum reached at a dP_T/dt value of about +4 mmHg/s. At higher rates the resistance curve levelled off. Similarly there was a graded active microvascular dilator response to inhibitory dP_T/dt stimuli (pressure decrease), maximal dilation being reached at dP_T/dt values of about -4 mmHg/s. Control microvascular resistance at normal P_T in this material averaged 6.0 ± 0.9 PRU corresponding to an average SVF of 51 ± 3 mmHg, SVP of 14 ± 2 mmHg and blood flow of 6.0 ± 0.2 ml/min/100 g tissue. The steady state (static) constrictor response to constant increase of P_T by 30 mmHg at control averaged 1.4 ± 0.2 PRU. In constructing the diagram of Fig. 2, we have assumed that the static constrictor response was fully developed at the peak of the initial constrictor component. This implies that the intercept of the dynamic constrictor response curve on the ordinate coincides with the static constrictor value, and that the dynamic dilator response curve starts from the initial control resistance level.

The results in Fig. 1 indicated that the microvascular resistance change per unit time, dR_{micro}/dt , was graded in relation to the dP_T/dt value, which might be another important aspect of dynamic myogenic regulation. This relation was therefore analysed in greater detail by determining dR_{micro}/dt at points where the slopes of the constrictor curves appeared to be virtually constant (cf. Fig. 1). The present deduction thus refers to the points where resistance in all cases exceeded the control value by 3 PRU. The corresponding analysis of the dilator curves (which were curvilinear) was made at the points where they were



graph illustrating the average dynamic myogenic microvascular constrictor and dilator responses made to increases and subsequent decrease in vascular transmural pressure (P_T) by 30 mmHg at different rates in the dP_T/dt range from -7.5 to 7.5 mmHg/s ($n=7$). The average static component to constant increased P_T by 30 mmHg above the control level is also shown.

resistance line. Fig. 3 summarizes the results for the whole material (7 expts). It can be seen that dR_{micro}/dt at the points of measurement was graded in relation to positive active values of dP_T/dt over a wide range.

As in Fig. 1 demonstrated that the microvascular constrictor response to dP_T/dt level off even when the dynamic stimulus was quite prolonged, but continued to be as long as the vascular bed was exposed to the stimulus. The peak response was

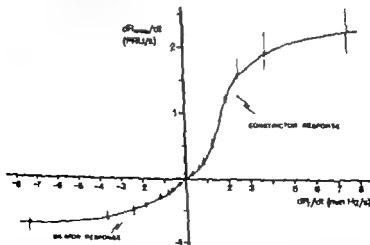


Diagram illustrating microvascular resistance change per unit time (dR_{micro}/dt) during increase and subsequent decrease in vascular transmural pressure (P_T) by 30 mmHg at different rates in the dP_T/dt range from -7.5 to 7.5 mmHg/s ($n=7$). For details see text.

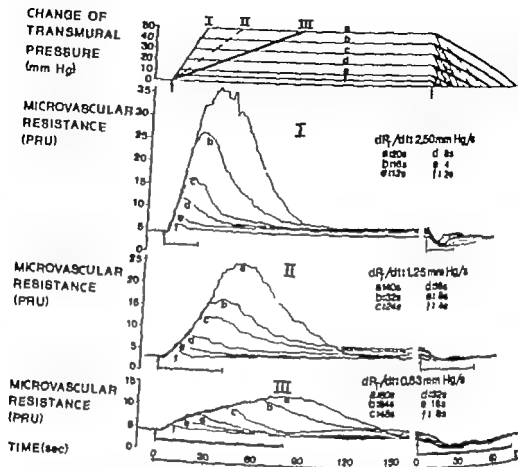
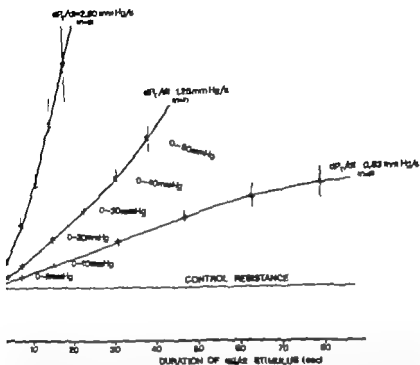


Fig. 4 Myogenic microvascular resistance responses evoked by graded increases in vascular transmural pressure, P_T , by 5 to 50 mmHg above control applied at three different rates (see key I-III) and by subsequent decreases in P_T at the same rates. The original consecutive resistance recordings are superimposed on top of each other. Note that the resistance responses at a given dP_T/dt value appear to be graded in relation to the amplitude of the P_T change and/or the duration of the dP_T/dt stimulus (see key signal in the curves indicates the longest duration of dP_T/dt stimulus in each series). The static, rate-independent constrictor responses are graded in relation to the amplitude of the P_T increase in the range from 5 to 40 mmHg.

thus reached shortly after the cessation of the dynamic pressure change which, in case of prolonged dynamic stimuli, seems to be an unusually long smooth muscle "contractile time" (> 48 s). This finding might have several explanations (see Discussion).

In an attempt to elucidate this problem microvascular resistance was observed when a vascular bed was exposed to P_T changes of different amplitudes in the range from 5 to 50 mmHg above control applied at three different rates, 0.63, 1.25 and 2.5 mmHg/s (Fig. 4). This approach also implied variation of the duration of the dynamic stimulus over a wide range up to 80 s (see key in Fig. 4).

It can be seen from this figure that the microvascular constrictor responses (plotted on top of each other as in Fig. 1) at each dP_T/dt value followed an almost identical slope during their development, reached their peaks not until shortly after cessation of the applied dP_T/dt stimulus, and were quantitatively graded in relation to the amplitude of the P_T increase (and the duration of the dP_T/dt stimulus). Calculated dR_{micro}/dt for each series (I-III) varied in a direct relation to the dP_T/dt value (cf. Fig. 3). Note also the distinct influence of dP_T/dt on



spiral data for the peak constriction response of the micro-vessels evoked by increases in vascular pressure as shown in Fig. 4. The constriction response to the three different dP_T/dt stimuli varies the duration of the dynamic stimulus which also applied simultaneous change of the static (see dotted lines). The dynamic myogenic constriction response seems to be influenced by both of the dP_T/dt stimulus and, indirectly by its duration, and by the amplitude of the P_T after expansion (see text).

Amplitude of the peak constriction response at each level of P_T change (5, 10, 20, 30, 40, mmHg). The delay from the onset of the dP_T/dt stimulus to the start of the constriction was almost identical at each dP_T/dt value, but was shorter the higher the dP_T/dt value, consistent with the results in Fig. 1. Resistance declined to steady state levels during and of constant increased P_T , and these static responses were graded in relation to the level of the P_T increase within the range from 5 to 40 mmHg (Fig. 4). When P_T was raised as much as 50 mmHg, the active static constriction response seemed to just barely offset the inherent passive vascular distension effect, implying an approximate return to the control level. The steady state response at each P_T level was of similar magnitude in panels I-III (like in the whole material) strongly indicating that it was in excess of the preceding dP_T/dt stimulus. The static transmural pressure stimulus, when raised (40 mmHg), increased resistance by no more than some 2 PRU above control level, nor, the static response was quite small compared to the up to 15 times larger dynamic response (Fig. 4). The effectiveness of the dynamic stimulus may be illustrated by the fact that a weak dP_T/dt stimulus of 0.63 mmHg/s at such a small P_T increase as 5 mmHg evoked a clear dynamic constriction response (Fig. 4, III D).

Decrease of P_T from any level applied at any rate depicted in Fig. 4 (right side) evoked active dilator responses in the microvessels. Mention should be made that dilator responses, in contrast to the constrictor effects, often reached their maximum at the cessation of the inhibitory dynamic stimulus (i.e., when the duration of the rise exceeded about 12 s). This difference, as will be discussed, may have important local implications.

Fig. 5 shows compiled data for the peak constrictor responses from the whole series of experiments of this type (cf. Fig. 4). The microvascular constrictor responses to the different dP_T/dt stimuli are here plotted versus the duration of the dP_T/dt stimulus. It also involves a simultaneous change of the P_T amplitude, illustrated by the dotted line. It can be seen that the peak constrictor response, at any of the three dP_T/dt values, with increasing duration of the dynamic stimulus and/or increasing amplitude of the rise. The responses were also clearly graded in relation to the magnitude of dP_T/dt . The data in Fig. 5 can be considered to reflect dynamic reactivity mainly since a static component possibly included in the peak response, is comparatively small.

It thus appears from Fig. 5 that the dynamic constrictor response is influenced both by the magnitude of the dP_T/dt stimulus (cf. Fig. 2) but also in some way by its duration and/or the amplitude of the P_T change (see Discussion). The relative quantitative contribution of the latter two factors is difficult to separate from each other. The following example from the diagram might illustrate the mutual interrelationship between these two factors. At a fixed P_T amplitude of 30 mmHg the dP_T/dt stimulus of 1.25 mmHg/s, lasting for 48 s, evoked a constrictor response of roughly the same magnitude as the dP_T/dt stimulus of 0.63 mmHg/s, lasting for 48 s. The dP_T/dt stimulus of 2.5 mmHg/s at the same P_T level was, however, much more effective, despite its short duration of 12 s. It thus appears that the magnitude of dP_T/dt , as expected, is the major determinant of the dynamic response.

All observations in this study were made on the acutely sympathectomized vessel to avoid adrenergic influence. There is some evidence in the literature, however, that constrictor effects to raised transmural pressure, in part, might be mediated by a local adrenergic nervous mechanism (e.g. Henriksen 1976). To investigate this possibility the dynamic and static microvascular effector responses were studied in several experiments before and after local α - and β -adrenergic blockade with phenoxybenzamine (15 mg/kg tissue, i.a.) and propranolol (1 mg/kg tissue, i.a.), respectively. Effective adrenoceptor blockade was achieved, as evidenced by complete abolition of adrenergic constrictor and dilator responses to large amounts (10 μ g/kg tissue, i.a.) of noradrenaline and isoprenaline. It is thus evident that the microvascular constrictor and dilator responses to transmural pressure changes were significantly attenuated by the adrenergic blockade, which strongly indicates that the described vascular reactions were myogenic in nature.

Discussion

The "microvessels" investigated in this study were delineated by the sites along the vessel tree from which small artery pressure and small vein pressure, via collaterals, were recorded. The dimensions of these vessels on the arterial side can be roughly estimated from data

as pressure and diameter measurements in the muscle microvessels reported by Zivkovich (1975). Our average value for small artery pressure of 51 ± 3 mmHg for the period thus indicates that this pressure was monitored from arterial vessels in the range of $15 \mu\text{m}$ and that the described vascular reactions therefore were mainly confined to vessels of this size and smaller. Minute venous vessels were also included in the estimate of microvascular resistance.

The present experimental approach to determine segmental vascular resistance bears a close resemblance to the basic principle for determination of pre-/postcapillary resistance advanced by Soto-Rivera and Soto-Rivera (1948). In the present study blood flow through all segments of the vascular tree, especially during the period of phasic shift in transmural pressure, is not necessarily exactly equal to arterial inflow owing to effects of passive vascular distension or recoil ("capacitance response") and net transcapillary fluid movement. Such effects are certainly encountered on the highly distensible venous side, and "large vein flow" in this type of investigation can only be approximately determined as (SVP - VP)/outflow (or instead of venous outflow arterial inflow minus recorded net fluid outflow and capacitance effects) as done in our previous study (Grünke *et al.* 1977). If flow is very rapid, SVP might even fall below VP for a few seconds and reversal in the veins would then have occurred unless it was prevented by the venous blood flow through the myogenically reactive arterial vessels, on the other hand, considered to be quite close to arterial inflow since passive distension caused by the rise in P is small here (Grünke *et al.* 1977) and in the overall microvessels negligible to judge from the absence of an initial decline of microvascular resistance during the period of increasing P (Fig. 1). Note in this context that if flow through arteries and minute veins in fact is somewhat smaller than arterial inflow during increase, and somewhat larger during decrease, of P we have, if anything, underestimated the effect of active reactions in the microvessels to transmural pressure stimuli, but the error must be relatively small.

The plethysmographic technique permitted all parts of the sympathectomized vascular bed of skeletal muscle to be exposed to strictly graded changes of vascular transmural pressure without alteration of extravascular (plethysmographic) pressure without affecting the venous pressure difference across the whole vascular bed. Vascular reactivity to graded transmural pressure stimuli could thus be studied without significant interference from the local chemical metabolic control system, since the induced changes of flow were quite small (for more detailed discussion see Grünke *et al.* 1977 *cf.* Johnson 1976). The described resistance responses to transmural pressure stimuli therefore must be myogenic in nature, a conclusion further corroborated by the facts that they were fully maintained after adrenoceptor blockade but completely abolished after papaverine administration.

In our previous publication (Grünke *et al.* 1977) evidence was obtained which strongly indicated the presence of a static as well as a dynamic component in myogenic microvascular control, an interpretation supported by the recent *in situ* studies on vascular smooth muscle mentioned in Introduction.

The present results provide additional evidence for this concept. The dynamic micro-

vascular resistance responses were thus found to be distinctly graded in relation to the magnitude of the dynamic stimulus, dP_T/dt , both for positive and negative values (Q_T and the static resistance responses, though small, were graded in relation to the magnitude of the applied P_T change over a wide range (Fig. 4). The microvascular resistance change per unit time (dR_{micro}/dt) was also directly dependent of the magnitude of the dP_T/dt stimulus, both for positive and negative values (Fig. 3). The dynamic myogenic control system thus bi-directional evoking constriction in response to excitatory stimuli (positive values dP_T/dt) and dilation in response to inhibitory stimuli (negative values of dP_T/dt).

The *in vitro* studies on isolated single-unit vascular smooth muscle mentioned in Introduction (Johansson and Mellander 1975, Sigurdsson *et al.* 1977) may help to explain the mechanisms behind these excitatory and inhibitory myogenic microvascular responses. Those investigations demonstrated the existence of clearly graded dynamic contractile myogenic responses to passive stretch at various rates (different dL/dt values) and that these responses were caused by graded facilitation of spike discharge primarily generated by "pacemaker" cells. Passive shortening at different rates caused relaxation by graded inhibition of normal pacemaker activity. The curve relating electrical (and mechanical) vascular smooth muscle responses to graded dL/dt values in these *in vitro* studies (Johansson & Mellander 1975, Fig. 3) shows conspicuous similarity to the present curve relating the microvascular effector response to dP_T/dt (Fig. 2) and to that relating dR_{micro}/dt to dP_T/dt (Fig. 1). It seems very likely, therefore, that the dynamic resistance responses *in vivo* are mediated via a similar myogenic pacemaker mechanism. If so, it appears that the dynamic *in vivo* facilitation or inhibition of spike discharge is a determinant of both the magnitude of the dynamic vascular effector response and its rate of development.

Dynamic stretch *in vitro*, especially when performed at high dL/dt , evoked an electrical afterdischarge and an attendant contractile response for some time after the cessation of the dynamic stimulus, apparently due to somewhat prolonged deformation of the receptor-unit (Johansson and Mellander 1975). Such an afterdischarge might at least partly explain why the peak microvascular constrictor responses were not reached until some time after the end of the dP_T/dt stimulus (Fig. 1).

A constant dynamic stimulus, when prolonged, would be expected to evoke a plateau constrictor response at the time the receptor-effector unit is maximally activated. A plateau of such an effect was occasionally noted, but only when the dP_T/dt stimulus lasted for 10 or more (Fig. 4 III, Fig. 5 lower curve). This, as mentioned, seems to be an unusually long "contraction time" for smooth muscle. The gradually increasing constrictor responses during prolonged dynamic stimulation at increasing P_T (Fig. 4 and 5) can only to a small extent be ascribed to a gradually increasing static response. It could be explained by very slow propagation of spikes from the receptor unit to a gradually increasing number of effector cells, but this seems unlikely in view of reported electrical propagation velocities of not less than 0.5–3 cm/s in other types of myogenically active vascular smooth muscle (Ljung *et al.* 1970). The dynamic receptor might in reality be sensitive to the rate of wall tension change, de/dt (see below). Wall tension can be calculated to increase gradually during the P_T change, dP_T/dt (see below). Wall tension can be calculated to increase gradually during the P_T change, dP_T/dt (by more than 100% at the peak of curve I a, Fig. 4) despite the counteracting effect of active lumen reduction. de/dt is more difficult to estimate, but rough deduction

ents that it also might increase gradually and, if so, could be a stimulus for the continuously increasing constrictor response. More likely perhaps, the described phenomenon can be explained by gradual recruitment of an increasing number of dynamic receptor units with different thresholds when P_T rises. The fact that resistance increased at an almost constant rate for a given prolonged dP_T/dt stimulus at gradually increasing P (Fig. 4) is in support of the latter hypothesis, since threshold phenomena in biological control are known to give rise to such effector characteristics (Licko 1973). The fact that the constrictor responses to negative dP_T/dt often reached their maxima before the cessation of the dynamic stimulus (Fig. 4) might then simply be explained by a small number of threshold units in operation at the preceding low level of vascular tone.

Presumably the dynamic myogenic receptor is probably not sensitive to dP_T/dt directly but might possibly be sensitive to length changes, dL/dt , provided the sensor element is placed in series with the contractile elements (see Johnson 1974), but perhaps more likely sensitive to changes of wall tension, as suggested from *in vivo* and *in vitro* observations (Johnson and Intaglietta 1976, Sigurdsson *et al.* 1977). The latter authors further stated that the dynamic myogenic responses to passive stretch were equally pronounced under conditions when vascular smooth muscle contraction manifested itself as active ("isometric" contraction) or as active shortening ("isotonic" contraction, like *in vitro*). The present investigation clearly demonstrates that active constriction and, hence, smooth muscle shortening *in vitro* does not result in abolition of dynamic myogenic reactivity. Rate-dependent regulatory mechanism is of great importance in most servo-control systems in order to increase rapidly stability and sensitivity (Melsa and Schultz 1969). The present data, taken together, indicate that the dynamic component in myogenic microvascular control serves such purposes. For proper function, most servo-control systems require a smoothly graded relation between the rate-dependent input signal and the resulting output signal, in this case corresponding to the dynamic stimulus and the effector response (i.e. frequency resistance change, and/or dR_{micro}/dt). Our results indicate that the dynamic myogenic microvascular control system also fulfils this criterion (Fig. 2, 3). Rate-dependent myogenic microvascular control was further shown to be bi-directional, evoking graded constrictor effects in response to positive, and graded dilator effects in response to negative, values of dP_T/dt . Such characteristics greatly increase the effectiveness of a servo-control system (Melsa and Schultz 1969). Bazar and Weiss (1969), however, reached the conclusion, from observations on the artificially perfused kidney of a uni-directional rate-sensitivity in myogenic control, but the absence of inhibitory dynamic effects in their study might possibly have been due to low vascular tone and reactivity in that particular preparation. It is possible that there are separate "receptors" for the static and dynamic stimuli in myogenic control (like in the highly differentiated muscle spindles of skeletal muscle), an assumption tentatively supported by the finding of predominant reactivity to static stimuli in the proximal arterial vessels and predominant reactivity to dynamic stimuli in the microvessels (Grände *et al.* 1977). The fact that strong inhibitory dynamic stimuli (high negative dP_T/dt) were able to cause profound decrease of vascular tone below the control level (Figs. 2, and 4.1) shows that they can interfere with the mechanisms responsible for normal basal myogenic tone in the microvessels. Dynamic stimuli might therefore contribute to

the very establishment of basal myogenic tone in the microvessels, for instance in pressure distension (Mellander and Arvidsson 1974 LaLone 1975). The great sensitivity and prompt reactivity of the microvessels to dynamic stimuli, demonstrated in this study, indicate that rate-sensitivity is of fundamental importance in normal microcirculation. It might here, by adjustments of precapillary sphincters and small resistance vessels contribute to various autoregulatory phenomena, for instance to autoregulation of capillary hydrostatic pressure accomplished by myogenic resettings of the ratio of pre- to postcapillary resistance (Järhult and Mellander 1974). No doubt, static myogenic stimuli are important for circulatory control (e.g. Baez, Laidlaw and Orkin 1974) and might, by its influence on proximal arterial vessels as well, be of special significance for local regulation of the overall resistance function in the vascular bed for instance in myogenic autoregulation of blood flow. A tentative theory for blood flow autoregulation based on repetitive dynamic myogenic stimulation of the vascular bed has been presented elsewhere (Mellander 1978).

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Nicotinic acid stimulates prostaglandin synthesis in the rabbit heart without releasing noradrenaline

By

LENNART KAUSER and ÅKE WENNEMÅLM

Nicotine is a powerful stimulator of prostaglandin (PG) synthesis in the rabbit heart (Wenmalm and Junstad 1976). The mechanism behind this synthesis stimulation is not known. Nicotine also elicits a short-lasting—“explosive”—release of sympathetic neurotransmitter (noradrenaline, NA) in this organ (Löfblom 1970). Since infusion of NA into the rabbit heart is known to stimulate its synthesis of PGs (Junstad and Wenmalm 1973) a possible mechanism behind the synthesis stimulating action of nicotine would be that it is mediated *via* the NA released. This would imply that the two effects of nicotine—release of NA and stimulation of PG synthesis—are closely associated.

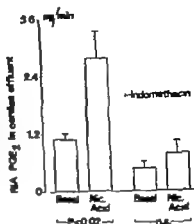
Nicotinic acid (NIC), which has the pyridine ring in common with nicotine, has also been suggested as a stimulator of PG synthesis in some different tissues (Andersson *et al.* 1977). It was therefore of interest to study firstly if NIC stimulates PG synthesis in the rabbit heart, and secondly if such stimulation occurs in parallel to a release of NA, or alternatively is dissociated from NA release.

Isolated rabbit hearts were perfused according to Langendorff with gassed and bubbled Tyrode solution. The effluent from the heart was collected and analysed for NA using the trihydroxy indole method. PGs in the effluent were adsorbed on Amberlite XAD-2 and eluted with ethanol. After evaporation the residue was quantified using a commercial kit for radio-immunoassay (RIA) of PGE (Clinical Assays Inc.) against a standard curve. NIC and indomethacin were added to the Tyrode solution to produce final concentrations of $2 \cdot 10^{-4}$ M and $5 \cdot 10^{-8}$ M, respectively. Perfusion of the heart was performed in 4 consecutive 10 min periods (I-IV) and effluent for analysis of PG and NA was collected during the last 5 min in each period. During I the heart was perfused with drug-free Tyrode solution and during II it was perfused with a solution containing NIC. During III indomethacin, a PG synthesis inhibitor (Vane 1971), was added to the Tyrode solution and during IV both NIC and indomethacin were present in the solution perfusing the heart.

Perfusion with NIC increased the basal outflow of RIA-PGE₂ by more than 150% (Fig. 1) compared to the basal outflow. No significant outflow of NA was found in the effluent neither at rest nor during perfusion with NIC. Heart rate and contractile force remained unchanged during perfusion with NIC. Perfusion with indomethacin decreased the

Fig. 1

Efflux of radio-steroidomethylated (RIA) PGE_2 from isolated rabbit heart perfused with drug-free (Basal) solution and its solution containing nicotinic acid (Nic. Acid, $2 \cdot 10^{-4}$ M), in the absence (the two left bars) and in the presence (the two right columns) of indomethacin ($5 \cdot 10^{-6}$ M).



low of RIA- PGE_2 by more than 50%. Subsequent perfusion with NIC, in the presence of indomethacin, did not significantly increase the efflux of RIA- PGE_2 (Fig. 1).

Comment

The current data demonstrate that NIC (in the absence of the PG synthesis inhibitor indomethacin) facilitates rabbit heart PG synthesis. Furthermore the data clearly show that stimulation of the PG synthesis by NIC occurs in the absence of release of NA. This implies that NIC stimulates PG formation directly probably by facilitation of the rate-limiting step in this synthesis, which is the mobilization of arachidonic acid from the tissue stores. Since ^{14}C stimulates PG formation by a direct action it seems, by analogy likely that nicotine—very closely related to NIC—facilitates PG synthesis via the same mechanism.

The present data support the concept that nicotine and NIC, by a direct action, stimulate biosynthesis of PGs. Such stimulation may be referred to the action of the common denominator of these compounds—the pyridine ring—on a receptive function in the myocardial cell membranes.

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The occurrence of somatostatin-like immunoreactivity in the vagal nerves

By

KERSTIN UVNÄS-WALLÉN, SUAD EFENDIĆ and ROLF LUTT

Electrical stimulation of the vagal nerves is followed by a release of somatostatin into the antral lumen of anesthetized cats (Uvnäs-Wallén *et al* 1977 a). The amount of somatostatin released in this way are quite small in comparison to the total amount of somatostatin. The release mechanism is probably not cholinergic since atropine does not block the release response (Uvnäs-Wallén *et al.*, to be published). Accordingly, it is suggested that electrical stimulation of the vagus might release somatostatin from and not from D-cells. In this connection, it is of interest that within the gastrointestinal tract somatostatin-like immunoreactivity has been localized to nervous structures (Uvnäs *et al* 1975) in addition to in endocrine-like cells. Furthermore, gastrin 17 is present in the vagal nerves, and a gastrinergic nerve plexus, confined to the distal part of the intestine and the colon, has been demonstrated by immunohistochemistry (Uvnäs-Wallén *et al* 1977 b).

For the above reasons it was investigated whether somatostatin-like immunoreactivity could be detected in the vagal nerves. For this purpose, pieces of the cervical, thoracic and abdominal vagi were collected from 6 anesthetized cats, and abdominal vagal specimens taken from 2 dogs. The biopsy material was immediately frozen on dry ice. After weighing, the frozen nerves were cut into small pieces and boiled for 20 min in water. After boiling, the volume of the extracts was adjusted to 10 ml and the content of somatostatin determined by radioimmunoassay (Elde *et al* 1977 a, 1977 b). The antibodies used in the present studies have been further characterized by Arimura (1977).

Somatostatin (80-600 ng/g) was found in the abdominal parts of the vagi from all cats, whereas smaller amounts (0-300 ng/g) were found in the cervical and thoracic parts of the vagal nerves (Table I). In the dogs, somatostatin-like immunoreactivity (17-40 ng/g) was found in the abdominal section of the vagal nerves (Table I).

At present we cannot state the origin of the somatostatin containing fibres in the vagal nerves. Most probably they come from vagal centres in the medulla oblongata. The accumulation of somatostatin in the distal part of the nerves supports this assumption since trace amounts of substances tend to accumulate in the distal part of the neurons.

The possible function of the vagal somatostatin containing fibres can only be speculated upon. These nerves may be involved in the well known regulatory functions exerted

TABLE 1. Concentration of somatostatin (ng/g) in different parts of the vagal nerves.

	Part of the vagal nerve		
	Cervical	Thoracic	Abdominal
1	0		90
2	0	20	300
3	0		120
4	111		80
5	30		400
6	100	300	600
7			17
8			40

nerve on gastrointestinal motility and tone as well as on HCl secretion. In this context, of interest that somatostatin inhibits HCl secretion (Bloom, *et al.* 1975, Le Roith *et al.* 1975, Raptis *et al.* 1975, Albarran *et al.* 1975) and gastrointestinal motor functions (Guillemin, Elondé and Mattsson 1977). Guillemin demonstrated that somatostatin reduced the force of the contractions of the longitudinal muscle of the guinea-pig ileum caused by electrical vagal stimulation, and that this effect was mediated by a decrease in the release of acetylcholine, indicating that somatostatin might act as an inhibitory modulator of cholinergic transmission (Guillemin 1977).

In contrast to somatostatin, gastrin has stimulatory effects on HCl secretion and on gastrointestinal tone and motility. Vizi *et al.* (1973) have shown that part of the myotonic effect, elicited by gastrin, is in fact caused by a release of acetylcholine from the Auerbach plexus.

Thus both gastrin and somatostatin can influence the release of ACh from ganglionic cells in the gastrointestinal tract (Vizi *et al.* 1973, Guillemin 1977). These findings, together with the demonstration of gastrin and somatostatin like immunoreactivity in the vagal nerves (Vizis-Wallensten *et al.* 1977 b) suggest that not only cholinergic fibres run in the vagi but also somatostatin and gastrin containing fibres, which may exert their actions by changing the release of acetylcholine from ganglionic cells. It is possible that selective activation of antagonistic vagal fibres may be one of the ways by which the CNS can control gastrointestinal functions, perhaps via modulation of cholinergic transmission.

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Inhibitory effects of noradrenaline and prostaglandin E₂ on neurotransmitter secretion evoked by single shocks or by short trains of nerve stimuli

By

LENNART SJÄRNE

amount of noradrenaline (NA) secreted per nerve impulse, as the result of electrical stimulation of adrenergic nerves, is highly dependent on the time interval between successive shocks (Hughes 1972) as well as on the number of impulses in each stimulus train (Hughes 1974).

The present paper is a preliminary report of studies of different factors modifying the releasing effect of repetitive impulses on the secretion of ³H-NA evoked by electrical stimulation of the postganglionic sympathetic nerves of guinea-pig isolated vas deferens (Sjörne, unpublished). It describes, firstly, an attempt to add to the previously available knowledge concerning the facilitating effect of repetitive stimulation, by using the measured secretion of ³H-NA per shock evoked by trains of 5-300 impulses to estimate the secretion caused by a single nerve impulse. Secondly it reports the effect of variation of the calcium level in the medium, and of interference with the 2 feed-back control mechanisms normally restricting secretory mechanism (for references see for example Sjörne 1973), on the secretion of NA evoked by a single nerve impulse, as well as on the facilitating effect of repetitive stimuli.

Guinea-pig vas deferens, in which the NA stores of the adrenergic nerves had been labelled by pretreatment with 2.4 μ M 11-(β)-NA, were superfused with modified Tyrode solution (calcium level adjusted to 1.8 or 3.4 mM), containing 20 μ g/ml sodium ascorbate, 0.6 μ M desipramine, 10 μ M normetanephrine, 1 μ g/ml atropine sulphate. The medium was aerated with carbogen gas (93.5% O₂, 6.5% CO₂). Temperature was 37°C and flow rate 2.5 ml/min. A Grass S44 Stimulator operated at 90 V 0.3 μ s, was used for brief postganglionic stimulation of the extramural nerves, via 2 platinum ring electrodes mounted 8 mm apart around the nerve trunk at its entry into the vas. The frequency of stimulation was 10 Hz. The number of shocks in each stimulation period was 300, applied either as one sequence, or subdivided into separate trains of 15 or 50 shocks repeated with 10 s intervals. The average fractional secretion of ³H-NA per shock was determined by dividing the rate of ³H efflux caused by the nerve stimulation in each period with the total amount of ³H (essentially equal to total ³H-NA) present at the time of stimulation. The value for Δt caused by a single nerve impulse was estimated by extrapolation from the average values for Δt caused by trains of 5, 15 and 50 shocks.

The results are shown in Fig. 1. Under each experimental condition Δt increased with the length of stimulus trains. A log-log plot of Δt against the length of stimulus trains formed a straight line for the Δt values obtained on stimulation with trains of 5, 15 and 50 shocks.

GUN EA P10 VAS DE ERE JS
FELD STIM 300 SHOCKS 10 Hz

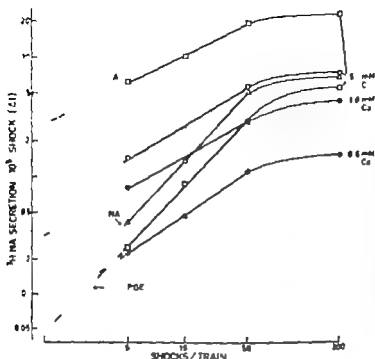


Fig. 1 Loglog plot of delta secretion of ^3H -NA per train (Δt) as a function of the number of shocks per train, at 5.4 mM calcium. Effects of 10 μM PGE and of 1 μM NA. Presence of 0.1 μM PGE. Experiments are carried out at 5.4 mM calcium. Each point represents the average of observations. Broken line is extrapolation to estimate single shock value for Δt .

Linear extrapolation from these values was used for tentative estimation of Δt caused by a single nerve impulse. Beyond a train length of 50 shocks a steady state was approached, apparently fully attained with trains of 300 shocks.

On stimulation in normal Tyrode (1.8 mM calcium) repetitive stimulation caused a 4-fold rise in Δt , from an estimated level of 0.3×10^{-3} of the total neuronal store of ^3H -NA to 1.2×10^{-3} at the 'fully facilitated' state. Stimulation with a single nerve shock, to 4.1×10^{-3} at the 'fully facilitated' state. Stimulation with trains of 300 shocks. Reduction of external calcium to 0.6 mM depressed Δt , and a 3-fold rise in calcium (to 5.4 mM) enhanced Δt , at all train lengths, including single nerve impulses. These results show that variation in external calcium concentration influenced the absolute values for Δt at all train lengths, but did not greatly affect the magnitude of facilitation due to repetitive stimulation. During the 9-fold change in external calcium (from 0.6 to 5.4 mM) the average value for $\Delta t_{300 \text{ shocks train}}/\Delta t_{\text{single shock}}$ was relatively similar 14.9 ± 4 ($n=3$).

Addition of 10 μM phentolamine (at 5.4 mM calcium) to remove α -adrenoceptor-mediated restriction of the secretory mechanism resulted in enhancement of Δt at all train lengths. The effect was inversely related to train lengths. $\Delta t_{300 \text{ shocks train}}/\Delta t_{\text{single shock}}$ was reduced 40%, to 7.7 compared with 12.8 at 5.4 mM calcium in the absence of phentolamine.

While addition of 1 μM NA (at 5.4 mM calcium) to stimulate the inhibitory α_2 -adrenoceptors did not significantly alter the 'fully facilitated' level of Δt , it did depress Δt caused by a single shock or by short trains of nerve stimuli. $\Delta t_{\text{single shock}}$ was enhanced 7-fold, to 90.3 compared with 12.8 at 5.4 mM calcium in the absence of NA. As seen in Fig. 1 the effect of 1 μM NA on Δt evoked by single shocks was equivalent with that of a 9-fold reduction in external calcium.

Effect of addition of 0.1 μ M PGE₂ in the presence of 10 μ M phentolamine (at 5.4 mM) was also inversely related to the length of stimulus trains. PGE₂ depressed the fully evoked level of Δt in the presence of phentolamine alone by 76%, but Δt evoked by a nerve shock by more than 98%. The ratio $\Delta t_{\text{nerve shock test}}/\Delta t_{\text{nerve shock}}$ was enhanced from 7.7 with phentolamine alone to 118 with phentolamine + PGE₂.

Present results show that the level of external calcium influences the absolute value for all lengths of stimulus trains, but not the magnitude of the facilitation caused by nerve stimulation. By contrast the 'auto-inhibition' of the secretory mechanism triggeredogenous NA, and the inhibitory effects of exogenous NA and PGE₂, influence both absolute levels of Δt at all train length (exception: exogenous NA did not significantly change the 'fully facilitated' level of Δt on stimulation at 10 Hz, but it has previously been shown to at lower frequencies, Stjärne 1973), and the magnitude of the facilitation caused by nerve stimulation. Their effects were thus inversely related to the length of stimulus trains. These findings appear to have practical implications for the interpretation of previous results obtained in guinea-pig isolated vas deferens. The particularly striking depressing effect of exogenous NA and PGE₂ on Δt evoked by very short trains of stimuli may thus explain the observation (Ambache and Zaar 1971) that exogenous NA depressed the contractile response to nerve stimulation with train of 5 shocks at 10 Hz, as well as the finding that PGE₂ depressed contractions caused by short trains (5-25 shocks), but less strongly (or not at all) depressed those evoked by longer trains (50 or more shocks) at 5-30 Hz (Euler and Pernow 1969; Ambache and Zaar 1971; Knoll *et al.* 1975).

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Cerebral sodium noradrenaline interaction: dipsogenic, antidiuretic and natriuretic effects

By

B. ANDERSSON, L. G. LEASELL, F. LESIAJKO and M. RUDOGREN

Intracerebroventricular (IVT) infusions of noradrenaline (NA), other monoamines, acetylcholine, have previously been shown to cause apparent release of antidiuretic hormone (ADH) in the hydrated goat (Olsson 1970, Vandeputte-Van Messom and Pottier 1971). Several investigators have found that these neurotransmitters may elicit drinking when injected into the hypothalamic region of the rat (cf. Fitzsimmons 1972). The dipsogenic responses have generally been regarded as due to transmitter effects at various synapses, and different, more or less elaborate models of mixed monoaminergic and cholinergic pathways in the cerebral thirst mechanism have been constructed. Another possibility would be that IVT administration of monoamines elicit thirst and ADH-release by acting on osmotic receptor-like receptors involved in the control of fluid balance. The striking sodium-noradrenaline interaction briefly reported here speaks in favour of this possibility.

Two female goats (b wt ca 35 kg) were used. The animals were maintained in positive sodium balance and had free access to water. They were supplied with permanent plastic triduum cannulas opening into the antero-dorsal part of the third cerebral ventricle. The construction of the cannula-system and the infusion technique have been described earlier (Åkerlund, Andersson and Olsson 1973). The IVT infusions (duration 20 min, rate 10 μ l/min) were made in the hydrated animal (90 min after the intraruminal administration of 100 ml/kg of 37°C water). Urine was collected via a retention catheter in 10 min samples for determination of flow rate, osmolality [Na⁺], and [K⁺]. The following IVT infusions were made at random order with a minimum interval of two days: a) NA (1-noradrenaline bitartrate 30 pmol/kg min) in either hypertonic (0.3 M) NaCl, isotonic (0.15 M) NaCl, or isotonic (0.3 M) d-glucose, and b) merely 0.3 M NaCl. Five experimental series including the four kinds of infusion were made. Two additional experimental series were also performed in which the dosage of NA was reduced to 7.5 pmol/kg min. Figures presented represent Mean \pm S.E.

Thirst. All infusions of NA/0.3 M NaCl ($n=7$) induced cumulative drinking in spite of the fact that the goats were hydrated at the start of the infusion. The latency time for drinking in response to the larger dosage of NA in hypertonic NaCl ($n=5$) was 9 ± 1.5 min with a total water intake of 1400 ± 80 ml. Also the smaller dosage of NA in 0.3 M NaCl induced drinking of >1000 ml of water during the second half of the infusion period. No drinking was consumed during any of the other three kinds of infusion.

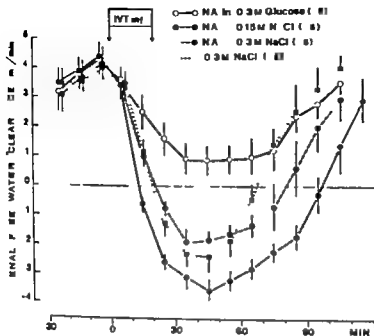


Fig. 1. Covalent interaction between sodium and noradrenaline (NA) in inhibiting the water diuresis of two infused goats. Dose of NA: $30 \mu\text{mol/kg min}^{-1}$. IVT inf = infusion into the third cerebral ventricle at $0 \mu\text{min}$. Vertical bars: S.E.

Antidiuresis. As shown in Fig. 1 the most pronounced antidiuretic effect was obtained in response to the NA/0.3 M NaCl infusions which induced negative renal free water clearance (C_{H_2O}) for about 90 min. In contrast, the C_{H_2O} generally remained positive after the NA/glucose infusions. Intermediary antidiuretic effects were obtained in response to the infusions of NA in isotonic saline and of merely 0.3 M NaCl solution.

Renal Na^+ and K excretion. The infusions of NA/0.3 M NaCl induced a pronounced natriuresis commencing 20 to 40 min after the infusion. The peak increase after the larger dose of NA was $370 \pm 50 \mu\text{mol/min}$ ($\sim 210\%$) with a simultaneous increase in renal K excretion of about 50%. The salturetic effect of the infusions of merely 0.3 M NaCl was roughly half that of the combined infusions (peak increases: Na: 110%, K: 30%). The rise in renal Na excretion induced by the infusions of NA in isotonic NaCl was insignificant (about 40%), and the NA/glucose infusions, if anything, temporarily reduced renal salt excretion.

Discussion. The integrated results of studies performed predominantly in the goat indicate that intraventricular sodium sensitive receptors play an essential role in the cerebral control of fluid balance, and suggest that Na⁺-transporting enzyme activity may be involved in the receptor excitation process (cf. Anderson 1977). The present study demonstrates that small amounts of NA administered into the third ventricle interact with cerebrospinal fluid (CSF) in eliciting thirst, apparent ADH-release, and natriuretics. An identical interaction with has previously been shown as regards angiotensin II, which has led to the sugges-

tion that angiotensin may elicit thirst and ADH release by affecting juxta-ventricular transport in a manner which stimulates sodium sensitive receptors (Andersson 1971). A similar mode of action appears possible also for NA. It would even become likely if adrenaline was found to interact with CSF Na⁺ in the same manner since both catecholamines have been shown to activate cerebral Na⁺-K⁺ ATPase (Desaiah and Ho 1977).

An approximate estimation based upon figures for CSF bulk flow in the goat (Pappenheimer *et al.* 1962) indicates that the lower dosage of NA used might have elevated the NA concentration in the third ventricle to 10^{-6} M. This level is too high to allow any speculation about eventual physiological importance of cerebral sodium-noradrenaline interaction in the control of fluid balance. However studies in progress will reveal whether the interaction can be demonstrated also with considerably smaller amounts of NA.

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Short-latency ventilatory responses to sudden withdrawal of hypoxia at normal and raised body temperature

In man

By

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Abstract

J. NORMANN, H. VEJBY-CHRISTENSEN and E. STRANGE PETERSEN. Short-latency ventilatory responses to sudden withdrawal of hypoxia at normal and raised body temperature in man. *Acta physiol. scand.* 1978. 102. 257-264.

Steady-state apnoeic conditions ($V_{E0}=40$ l/min) were achieved by the inhalation of asphyxial gas mixtures (P_{A,O_2} 60 torr, P_{A,CO_2} 40-45 torr) in normothermia and after a rise in rectal temperature of $^{\circ}\text{C}$ had been induced by heated flying suit. Arterial chemoreceptor drive was transiently reduced by subsequent removal of hypoxia (type (1) tests: two breaths of CO_2 in O_2) or simultaneous withdrawal with hypercapnia and hypoxia (type (2) tests: two breaths of O_2). 8-13 tests of each type were performed with apnoeic conditions in 6 experiments on 4 healthy human subjects. Expired volume, total breath duration and expiratory time were recorded, and minute ventilation and expiratory time subsequently computed as by breath. In hyperthermia the steady-state ventilation of 40 l/min (at relatively longer respiratory time and correspondingly lower tidal volume) was achieved at P_{A,CO_2} each was 5 torr lower than normothermia. Ventilation decreased significantly in all tests. Tested with 3-way analysis of variance significant differences between the ventilatory responses at the two temperature conditions, and between the test types were found. The rate of change of ventilation is greater in hyperthermia than in normothermia, and also greater in type (2) tests than in type (1) tests. Since apnoeic conditions existed prior to the tests, this implies that the arterial chemoreceptor contribution to the total ventilatory drive is less in hyperthermia. In type (2) tests significant lengthening of expiratory time as observed in the first breath. This finding confirms the effect in man of changes in arterial P_{CO_2} on long stretch receptor firing.

It has long been recognized that a rise in body temperature in man is associated with an increased ventilation (Haldane 1905, Cunningham and O'Riordan 1957). Previous studies of the steady state ventilation in man have indicated that this is due to effects on both central regulatory mechanisms and arterial chemoreceptors (Vejby-Christensen and Strange Petersen 1973 a, Strange Petersen and Vejby-Christensen 1977).

It is possible to study the contribution of the arterial chemoreceptors in man directly by isometric approaches. Inhalation of an asphyxial gas mixture is associated with strong stimulation of the arterial chemoreceptors. As shown by Bouverot *et al.* (1965) and exploited

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in man by Miller *et al.* (1974) it is possible to reduce chemoreceptor activity (perhaps silence them) by transient withdrawal of either CO_2 , hypoxia, or both simultaneously.

The present experiments were designed to provide further information about the role played by the arterial chemoreceptors in the control of breathing during hyperthermia in man by studying the dynamic, short latency ventilatory responses to changes in arterial tensions. Transient relief of a moderately strong asphyxial stimulation of the arterial chemoreceptors was achieved either by the simultaneous removal of both the hypoxic and hypercapnic stimuli, or by isocapnic removal of the hypoxic stimulus.

Methods

Four healthy young men (aged 19–22 years) were studied. Two of them were medical students and had knowledge of physiology but none of them were informed about the details of the study. Only the effect of body temperature on breathing during the inhalation of various gas mixtures. All subjects signed a consent form before the first experiment.

Each exp't consisted of two identical sessions, one before and one after rectal temperature had been raised by approximately 1.6°C. Two subjects took part in 4 exp'ts., and two in 1 exp't. each. The subjects were encouraged to read throughout the experimental periods.

Breathing apparatus

The basic open-circuit employed was like that of C. Cunningham, Howson and Pearson (1970). The subject breathed warm, humidified air from one of two gas-supply lines through a modified Lloyd valve. During the two lines was performed during expiration by the operation of remotely controlled solenoid valves the subject was unaware of when switching was performed. The subjects expired through a condenser and a dry gas meter (CD4 Parkinson & Cowan Ltd, London) into a low-inertia wedge spirometer (Oxford Instruments, Oxford) which was emptied during the following inspiration. The dry gas meter provided a quickly available index of mean ventilation which was especially suitable in experiments involving isopneustic comparisons. Tidal volume (V_T) was determined by integration of the output from the wedge spirometer. Testing with sinusoidal pump the output of the wedge spirometer as found linear in the range of flow from 5–300 L/min. Inspiratory, expiratory and total breath durations (T_I , T_E and T_T) were determined from the changes in mouth pressure measured by a sensitive manometer (MDC 90, Hilger I.R.D. Ltd, London) they were recorded as large-time ramp reset at the beginning of inspiration and interrupted briefly at the transition from inspiration to expiration (Fig. 1). End-tidal $\text{P}_{\text{a}}\text{O}_2$ and $\text{P}_{\text{a}}\text{CO}_2$ were measured with a mass spectrometer (MM 201 V.G. Micromass Ltd, Wimsford) sampling at the mouth. Calibrations with standard gases previously analyzed on the Lloyd-Haldane apparatus were performed with regular intervals during the experiments. The respiratory variables are recorded by multi-channel hot-stylus recorder (MS Devices Ltd, Welwyn Garden City, England), and the data were extracted from the records using a P.C.D. trace reader (P.C.D. Ltd, Farnborough, Hants, England).

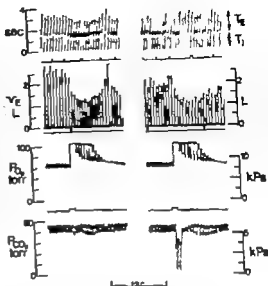
Heating procedure

The subjects were dressed in a heated flying suit (Junex Electrics Ltd, Glasgow). Innermost they wore light shirt and trousers, and over that a polyethylene suit with constrictions at the wrists and ankles to prevent evaporative heat loss from the trunk and extremities. Over that was the actual heating suit which had heating elements on the front and back of the trunk and around the extremities. Insulators and wires with heating elements were used periodically. Outside the heating suit the subjects wore an insulating suit with hood and with muffs at the wrists and ankles. The heating suit was supplied with current from four 12 V batteries through rheostat and an ammeter. By controlling the current it was possible to induce a rise in body temperature and to maintain the temperature within narrow limits of the desired level during the hyperthermic part of the experiments. The heating-up period lasted about 1 h, and the mean rectal temperature rise in the exp'ts. was 1.66°C (Table 1). During the warming-up period the subjects were given salted fruit juice (400–600 ml) to replace the losses through sweat production.

Temperature measurements

Rectal temperature was measured with a thermocouple placed at a depth of 10 cm from the anus and 1 cm from the rectal wall (Copenhagen).

we specimen of experimental record
 left as example of type (1) test
 removal of hypoxia), on the right
 test (simultaneous removal of both
 and hypercapnia). From above down:
 breath duration (t) split into T (below
 line) and T_E (above break); marker
 sector, signals indicating the passage
 expiratory volume V_E (L), the increas-
 ing $V_{T,E}$, P_{O_2} and P_{CO_2} at the smooth
 ed by even marker showing the time
 lack test gas was added. The P_{O_2}
 traces lag behind the others by
 delay of about 1.5. Note that the
 P_{O_2} does not return to the steady
 val immediately after the test. This is
 the fact that the space between the
 the end the solenoid valve in the test
 pply less after its closure is poorly
 and space (cf. Cunningham *et al.* 1973
 8).



Material procedure

The procedure employed in the present study was to replace steady apyral mixture with oxygen (O_2 in ex) gas) for two breaths. A moderately strong arterial chemoreceptor drive (P_{A,CO_2} 60 torr (8.0 kPa), P_{A,CO_2} 40–45 torr (5.3–6.0 kPa)) was induced by giving the subjects gas mixture with P_{O_2} about 100 torr (13.3 kPa) and P_{CO_2} of 31–36 torr (4.2–5.1 kPa). We attempted to achieve isoeupnoic conditions (i.e. ventilation of approximately 40 L/min) in the steady state at each of the two temperature levels by means of the inspired CO_2 concentration. This gas mixture was breathed for at least 15 min before the test was performed. At intervals of at least 3 min between each two different types of test we performed Test type (1), isoeupnoic removal of hypoxia, the test gas being oxygen with CO_2 added so as to maintain an unchanged P_{A,CO_2} during and after the test. Test type (2), simultaneous removal of hypoxia and hypercapnia, the test gas being oxygen. In both test types the test gas was given for two breaths (breath and breath 1) after which the subjects were switched back to the steady-state mixture with smaller amounts of the CO_2 concentration in order to maintain P_{A,CO_2} at the same level as before the test. P_{A,CO_2}

For each of the two test types at the two temperature conditions studied in each experiment are shown: initial temperature ($^{\circ}C$), mean pre-test P_{A,CO_2} (torr), maximal P_{A,CO_2} change with test, and mean pre-test breath duration (T_E , sec) and ventilation (V_E , L/min).

Temp	Test type (1)					Test type (2)				
	P_{A,CO_2}	$\Delta P_{A,CO_2}$	T	V		P_{A,CO_2}	$\Delta P_{A,CO_2}$	T	V	
36.30	43.4	11.8	3.06	37.3		43.8	13.9	3.03	36.3	
37.70	42.2	9.2	2.61	45.9		43.0	13.2	2.65	43.5	
36.45	42.6	0.3	1.76	40.8		43.1	14.6	1.82	40.0	
37.65	39.1	0.6	1.32	42.6		39.4	11.2	1.38	41.6	
36.15	44.5	0.3	1.98	37.3		44.4	16.8	2.00	37.8	
37.80	39.8	0.6	1.61	39.7		40.8	12.5	1.63	39.6	
36.20	43.2	0.3	3.31	38.2		43.1	12.1	3.32	38.1	
36.18	37.7	0.9	2.80	37.0		37.6	7.9	2.78	36.0	
36.18	46.2	0.6	3.49	39.3		46.1	14.4	3.32	38.3	
37.40	37.8	0.7	2.56	37.9		38.2	7.4	2.83	36.4	
37.15	46.3	1.6	2.51	42.1		46.5	10.9	2.79	40.6	
36.65	39.4	11.8	2.20	40.8		39.4	7.1	2.79	40.2	

TABLE II Three-way analysis of variance of the effects of A. temperature, B. test type and C. breath (0-4) on minute ventilation calculated breath by breath.

	434.12	462.2	462.3	411.7	411.1
A	+++	++	NS	+++	++
B	++	++	+++	+++	++
C	+++	+++	+++	+++	+++
A B	NS	++	+	NS	NS
A C	NS	NS	++	NS	NS
B C	NS	NS	++	NS	NS
A B C	NS	NS	NS	NS	NS

+++ = $p < 0.001$ ++ = $p < 0.01$ + = $p < 0.05$ NS = $p > 0.05$

increased in both test types to more than 200 torr (27.0 kPa) in breath 0. $P_A \text{CO}_2$ fell by 10-14 torr (1.3 kPa) in type (2) tests (cf. Table I). The sequence of the tests were randomized, each exp. compared to thirteen successful tests of each type in each temperature condition.

Results

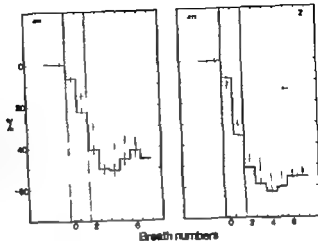
Fig. 1 shows two segments of an original record (expt. 411.7), on the left a type (1) test and on the right a type (2) test. It shows the steep rise and subsequent slow return of $P_A \text{CO}_2$ and for $P_A \text{CO}_2$ the approximate constancy in type (1) and the drop in type (2). The associated changes in expired volume (V_E) are obvious. V_T , T_I and T_E were measured for breaths -1 0 1 2 3 4 5 6 and 7 and expired minute ventilation (V_E) was subsequently calculated for each of these breaths.

In the statistical treatment of the data a 3-way analysis of variance (Sokal and Rohlf 1969) was performed. The number of tests in each experiment was equalized (3-4) by exclusion of tests using random numbers. The analysis was carried out on the values of minute ventilation of breaths 0, 1, 2, 3 and 4; breaths 5, 6 and 7 were not included in this analysis since only ventilatory changes occurring within 10-15 sec of a test (Rohlf *et al.* 1965; Miller *et al.* 1974) are indicative of relief of arterial chemoreceptor drive. In this three-way analysis of variance the effects of body temperature, test type and breath number were tested. The results are shown in Table II. Significant effects related to all three factors were found in all experiments with the exception of temperature in experiment 462.3. The effect of breath number merely reflects the temporal pattern of the response (Fig. 1). In all but one expt. a rise in body temperature was thus found to cause a significant ventilatory response to tests of both types, and in all expts. the responses to the two test types were different at both levels of body temperature.

Fig. 2 shows the average changes in ventilation as a function of breath number before and after both test types in both temperature conditions in expt. 411.7 which largely confirm the general findings. It is seen how \dot{V}_E decreased following the tests, and how for both test types the responses were larger in the hyperthermic than in the normothermic condition. Comparing the two test types further shows that the responses in both temperature conditions were larger following tests of type (2) than of type (1).

As stated in methods it was intended that the steady-state ventilation in both temperature conditions was the same, 40 l/min. This was achieved

Mean percentage changes in ventilation during and pe (1) tests (left) and type (right) at normal (broken elevated body temperature) in expt 411.7. The 1 area corresponds to the rate (2 and 1) during which 1 gas is inhaled.



e 1) in all but 1 expt. (434.12), where the ventilation in hyperthermia was some 8-9% higher than in normothermia. However an analysis of variance performed on the % of changes in ventilation from the control values (the means for breaths -2 and -1) led also in this expt. significant effects of both temperature and test type on the results.

In attempt to quantitate the differences due to test type and to body temperature the mean slopes of the absolute values of minute ventilation on breaths numbers 0 to 4 calculated (Table III). The slope thus calculated is an index of the mean fall in ventilation per breath during and after tests. It was numerically greater in hyperthermia than in normothermia in 10 out of 12 cases (the exceptions being 462.2 in type (1) tests and 411.8 type (2) tests). The mean difference in slope was 1 l/min/breath. Comparison of the two types showed larger slope values in type (2) tests in 9 out of 12 cases (the exceptions being 462.2 in normothermia and 411.8 and 440.4 in hyperthermia). The mean difference in slope was 0.5 l/min/breath.

The transient ventilatory responses were largely due to decreases in tidal volume; respiratory frequency usually remained constant although in some tests either a small decrease

TABLE III Regression analysis of ventilation (calculated for individual breaths) against breath numbers 0-4. $V = b$ breath number. a . Only slope slopes (b , l min⁻¹ per breath) are shown.

Expt.	Normothermia		Hyperthermia	
	Type (1)	Type (2)	Type (1)	Type (2)
	b	b	b	b
412	0.8	1.7	-0.7	3.7
22	2.3	1.6	-1.7	-2.7
17	1.9	2.7	2.8	3.4
118	3.6	-4.4	4.4	-4.9
	3.4	-4.9	4.3	4.4
	3.1	-4.3	6.3	5.1

(-lengthening of T_T cf Fig. 1 right) or less commonly a small increase (shortening of T_T cf Fig. 1 left) was observed. A detailed analysis of the respiratory pattern in the steady state and during the transient responses revealed results which are largely confirmed by previous findings; these results will therefore not be presented here.

Discussion

At normal body temperature the results obtained in the present study regarding time course and magnitude of the ventilatory responses to tests were largely comparable to previously published data from this laboratory (Miller *et al.* 1974; Cunningham, Drysdale and Miller 1976). This agreement like arguments presented elsewhere (Strange Petersen and Vejbjerg-Christensen 1977) would argue against any unspecific effects associated with the test suit or apprehension in anticipation of the "warm" session affecting the responses.

For both test types, however, the analysis of variance showed significant differences in responses following the induction of a 1.6°C rise in rectal temperature. The quantitative estimate obtained by the regression analysis (Table III) revealed larger ventilatory responses in hyperthermia than in normothermia in 5 out of the 6 expts.

It should be emphasized that the steady-state P_{A,CO_2} was different in the two temperature conditions. In normothermia the ventilation of 40 l/min was achieved at a P_{A,O_2} of 44.8 torr (6.0 kPa) and a P_{A,CO_2} of 44.8 torr (6.0 kPa). In hyperthermia at a P_{A,O_2} of 60 torr (8.0 kPa) and a P_{A,CO_2} of 39.4 torr (5.2 kPa) (mean values, cf Table I). For the overall control of ventilation it was thus found that a rise in body temperature of 1.6°C has the same effect as an increase of P_{A,CO_2} of ca. 5 torr (0.7 kPa). Similarly in tests of type (2) the ratio $\Delta \dot{V}_E / \Delta P_{A,CO_2}$ was larger in hyperthermia than in normothermia.

Inspection of Fig. 2 shows decreases in ventilation in breath 0, such early changes being most commonly observed in type (2) tests. They occurred too early to be arterial chemoreceptor responses and can probably partly be explained by the increase in blood density in the high O_2 test breaths. In type (2) tests a lengthening of the expiratory time (T_E) was observed in breath 0; this confirms findings published and discussed elsewhere (Cunningham *et al.* 1977) and provides further evidence that interaction between stretch receptor activity and airway P_{CO_2} (Mustafa and Purves 1973; Bradley, Noble and Trenchard 1976) is present and active in man. We found the lengthening of T_E in normothermia ($80 \text{ ms} \pm 22$, $n=63$) than in hyperthermia ($50 \text{ ms} \pm 30$, $n=68$); this might be due to a greater change in P_{A,CO_2} in normothermia than in hyperthermia (about 10 versus about 5 torr). Consequently these findings do not permit any conclusions as to whether the stretch receptor- P_{CO_2} interaction is modified by changes in body temperature (see below). The lengthening of T_E (and T_T) contributed to the observed reduction in ventilation in breath 0 in type (2) tests. In type (1) tests, on the other hand, only a small and insignificant shortening of T_E was found in breath 0 in either temperature condition.

In both normo- and hyperthermia the ventilatory responses to tests of type (2) were consistently larger than those to type (1), a finding which agrees with that of a previous study on man (Miller *et al.* 1974). It seems likely that this difference may in part be due to the dynamic response properties of the chemoreceptors. The response to the

Oskey and Torrance (1971) observed no transient over or undershoot in the carotid nerve activity upon induction or relief of hypoxia (cf. our test type (1)) whereas relief of hypercapnia alone (cf. our test type (2)) caused a transient decrease to zero activity. A main finding of the present study was that the ventilatory response to tests of either as larger in hyperthermia than in normothermia, significantly so in 5 of the 8 expts. short latency reduction in ventilation following an O_2 -test is due to a fall in the input from the arterial chemoreceptors to the respiratory centre. Since the present comparison is based upon virtually isopnoeic conditions in the steady-states between tests, the larger responses found in hyperthermia indicate that the contribution to ventilation attributable to arterial chemoreceptors was also relatively increased in this state. Whether or not a direct interaction between peripheral and central chemoreceptor input takes place, this implies that that part of the total ventilatory drive which depends upon peripheral chemoreceptor activity is increased in hyperthermia. These results thus confirm our previous steady-state findings of an enhanced ventilatory response to hypoxia in hyperthermia (Vejby-Christensen and Strange Petersen 1973 a, Strange Petersen and Vejby-Christensen 1977) and are consonant with the contention that this is due to an increased arterial chemoreceptor activity. Evidence from animal work that this is the case has most recently been presented by McQueen and Eyzaguirre (1974).

The hyperventilation associated with rise of body temperature in the absence of hypoxia (Adams 1905, Cunningham and O'Riordan 1957, Strange Petersen and Vejby-Christensen 1973) where peripheral chemoreceptor contribution is believed to be small, suggests that hyperthermia also affects intracranial chemosensitivity.

A simple, unspecific sensitization of "respiratory centre" neurons caused by a rise in temperature, on the other hand, could hardly explain why the increase in ventilatory response to hypoxia produced by a certain elevation of the body temperature is relatively larger than the increase in ventilation produced by the same temperature elevation (when air is inspired). Our previous finding, that the magnitude of the fast ventilatory response at the onset of hypoxia was unaffected by hyperthermia (Vejby-Christensen and Strange Petersen 1973 b) also argues against such unspecific sensitization. More direct evidence against this idea, recently has been presented by Chai, M. and Brobeck (1965) and by Tabatabai (1972) who found decreases in both tidal volume and respiratory frequency in response to localized heating of areas at the medulla corresponding to the respiratory centres.

Besides affecting the intracranial and peripheral chemosensitivity rise in body temperature has also a third, direct effect on the pattern of breathing, which has been fully confirmed in all steady-state measurements of ventilation, breath duration and tidal volume in the present study (Table 1). It affects the ponto-medullary mechanisms responsible for the setting of respiratory rhythm so that a given ventilation is achieved at a relatively higher frequency and a correspondingly lower tidal volume (Hey *et al.* 1966, Euler, Herrero and Weder 1970, Euler and Trippenbach 1976, Strange Petersen and Vejby-Christensen 1977).

Some authors (Schoener and Frankel 1972, Grunstein *et al.* 1973), finally have reported evidence of a fourth effect, a rise in temperature causing an increase in the vagal feedback from stretch receptors in the lungs. As briefly indicated above our present data do not provide any evidence as to the importance of this in man.

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The mechanism of histamine release induced by the ionophore X537A from isolated rat mast cells

I. Significance of Monovalent Cations, Calcium, Metabolic Energy and Temperature

By

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Abstract

KAZMIERCZAK, W., S. A. PATKAR and B. DIAMANT. The mechanism of histamine release induced by the ionophore X537A from isolated rat mast cells. I. Significance of monovalent cations, calcium, metabolic energy and temperature. *Acta physiol. scand.* 1978. 102. 265-273.

Histamine release induced by X537A from isolated rat mast cells was dependent on the presence of sodium in the incubation medium. The rate of release was faster in potassium medium, but the final release was the same after prolonged incubation. Extracellular calcium as well as metabolic inhibitors depressed the rate of release induced by X537A. Preincubation of mast cells with EDTA or the ionophore A23187 in the presence of extracellular calcium did not influence subsequent release induced by X537A. The findings of release at 45°C and lack of release at 0°C distinguish the action of X537A from calcium- and energy-dependent releasing agents like compound 48/80 and A23187 on the one hand and the simple lytic action of dectylamine on the other.

Amphoteric antibiotics A23187 (Eli Lilly & Co.) and X537A (Lasoloid) (Hoffmann-La Roche) are able to transport cations across biological membranes (Cawwell and Pressman 1973, Reed and Lardy 1977a and b, Pressman 1973). They have been used in recent years to study the involvement of calcium and other ions in various secretory processes. A23187 is a more specific carrier of calcium than X537A, which in addition has been shown to transport monovalent cations as well as some primary amines (Pressman 1973). Furthermore, X537A has been shown to exert a greater depolarizing action than A23187 on frog skeletal muscle fibers (Cochrane and Douglas 1975). Therefore, some of the functional properties observed for X537A might be ascribed to other effects than to an enhanced transport of calcium. The dependence of X537A on calcium for histamine release from isolated rat mast cells has been a subject of conflicting opinions (Foreman *et al.* 1973, Cochrane and Douglas 1974, Kagiyama and Douglas 1974). The present investigation was performed in order to clarify the mechanism by which X537A elicits the release of histamine from isolated rat mast cells.

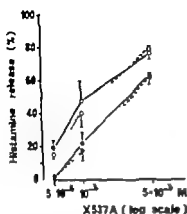


Fig. 1

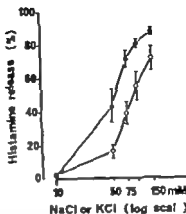


Fig. 2

The inhibitory action of calcium (1 mM) on histamine release induced by X537A. Mast cells were exposed to increasing concentrations of X537A in the presence (filled symbols) or absence (open symbols) of calcium. The cells were either preincubated with calcium for 10 min and the reaction started with X537A (dotted lines), or X537A was preincubated together with calcium and the reaction was started with cells (continuous lines). Incubation time: 10 min. Each point represents mean \pm S.E. of 3-4 expts.

2. The influence of increasing concentrations of NaCl (open symbols) or KCl (filled symbols) on histamine release induced by X537A (10^{-6} M). After isolation mast cells were washed twice with Tris-Acetic with Tris-glucose before exposure to X537A in Tris-sodium or Tris-potassium. Mean \pm S.E. of 3-4 expts.

amine content (Fig. 1). At each point investigated, the presence of 1 mM calcium decreased the release, irrespective of whether the cells were preincubated with calcium for 10 min prior to the addition of X537A or whether the reaction was started by the addition of X537A to the incubation medium containing X537A and calcium. It was further observed that suboptimal release induced by 10^{-8} M of X537A after 10 min gradually increased during prolonged incubation, also in the presence of calcium, indicating that calcium decreased the rate of the release process (data not shown).

In 6 expts. performed in Tris-glucose, X537A was found to be ineffective to release histamine above the spontaneous release after 10 min of incubation. In the same expts., when Tris-glucose was gradually substituted with increasing concentrations of calcium up to 110 mM, no histamine was released by X537A (10^{-6} M). However in Tris-calcium (110 mM) as well as in Tris-glucose the spontaneous release was elevated to $29.3 \pm 2.8\%$ and $17.1 \pm 1.2\%$, respectively as compared with $6.5 \pm 0.7\%$ in Tris-sodium (150 mM).

When mast cells were preincubated for 2 h at 37°C in Serensen-A before exposure to calcium (1 mM) and compound 48/80 or to compound 48/80 alone, the secretory response actually decreased. When EDTA (1 mM) was present during the preincubation period a further decrease of the release was found after challenge with 48/80 alone, but not after simultaneous addition of compound 48/80 and calcium (Table I). In contrast, the response of mast cells to X537A in the absence of calcium was unaffected by pretreatment of the cells with EDTA (1 mM) for 2 h. Furthermore, pretreatment of mast cells in Serensen-A with the antihistamine A3187 (10^{-6} M), which completely abolished histamine release induced by

TABLE I The influence of EDTA (1 mM) on histamine release induced by compound 48/80 and X537A (10^{-6} M)

Preincubation	Histamine release (%)			
	48/80 (n = 7)	P _{diff}	X537A n = 3	
	-Ca	+Ca	Ca	
3 min	43.4 ± 5.7	63.8 ± 4.8	< 0.001	56.0 ± 11.5
2 h	30.4 ± 3.0	46.6 ± 4.4	< 0.01	48.2 ± 6.6
2 h with EDTA (1 mM)	16.4 ± 3.4	49.4 ± 3.3	< 0.001	48.2 ± 1.6

Following preincubation in Sorensen-A at 37°C as described in the table the cells were added into Sorensen-A containing 48/80 or X537A and eventually calcium (1 mM). Incubation is then for 10 min. P_{diff} 48/80 3 min 2 h (-Ca) <0.05 3 min 2 h (+Ca) <0.01 2 h EDTA (-Ca) <0.001

compound 48/80 did not influence histamine release induced by X537A or de (Table II)

The influence of monovalent cations on histamine release induced by X537A

When mast cells were incubated in Tris-sodium or Tris-potassium containing in concentrations of the monovalent cations and exposed to X537A (10^{-6} M) for histamine release was evident in the presence of 50 mM of either ion. The release by increasing their concentration and at each concentration the release observed in the presence of potassium was markedly higher than that obtained with sodium (Fig. 2). Correspondingly incubations with X537A ($7.5 \cdot 10^{-6}$ M) for various periods in Tris-p (150 mM) and Tris-sodium (150 mM) showed that the rate of release was faster in the potassium medium, but the total release after prolonged incubation was the same (80%) in both (Fig. 3).

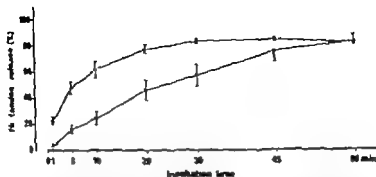
The influence of temperature

When mast cells were preincubated in Sorensen A for 10 min at different temperatures before the addition of compound 48/80 (0.6 µg/ml), X537A (10^{-6} M) or A23187 (together with calcium (1 mM) none of the agents released histamine at 0°C (Fig. 1). In this respect they differed from decylamine (30 µg/ml), which in 4 experiments released histamine equally well at 0°C as at 37°C (data not shown). The release induced by X537A increased with the incubation temperature and was more pronounced at 45°C.

TABLE II The influence of pretreatment of mast cells with the ionophore A23187 on subsequent release induced by compound 48/80 (0.6 µg/ml), X537A (10^{-6} M) and decylamine (25 µg/ml)

Reagents	Histamine release (%)	
	Control cells	Ionophore-treated cells
48/80	43.9±1.3	0.6±0.4
X537A	65.9±11.7	66.3±10.3
Decylamine	79.5±12.4	80.3±12.4

Mast cells were preincubated with 10^{-8} M of A23187 for 3 min (Diamant) and then incubated for 10 min in the presence of releasing agents. Mean



The time course of histamine release induced by X537A in Tris-sodium (130 mM) (open symbols) and Tris-potassium (150 mM) (filled symbols). After isolation mast cells were washed twice in Tris-A and Tris-glucose and then incubated concentrated in 100 μ l Tris-sodium or Tris-potassium containing $7.5 \cdot 10^{-4}$ M for increasing lengths of time. The reaction was stopped by transferring 20 μ l of it to 2 ml ice-cold Tris-glucose. Each point represents mean \pm S.E. of 4-6 expts.

A23187 and compound 48/80 effectively released histamine at both 21°C and 37°C, virtually no release was induced by these agents from cells incubated at 45°C. After 10 min of incubation, X537A released less histamine at 21°C than the other two releasing agents. However, by prolonging the incubation time at 21°C, the release induced by X537A gradually increased, so that after 90 min the release amounted to the same values as observed at 37°C (97% of the total histamine content, data not shown).

Influence of metabolic energy

Mast cells were incubated in Serenzen-A together with 10^{-4} M of Antimycin (an inhibitor of oxidative phosphorylation) for 10 min prior to the addition of various concentra-

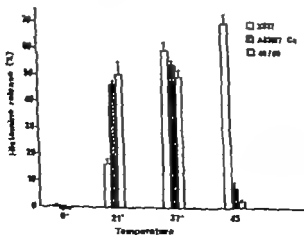


Fig. 4 The influence of temperature on histamine release induced by compound 48/80, X537A or A23187 in the presence of calcium. Mast cells were isolated and preincubated in 100 μ l Serenzen-A for 10 min at each temperature before the addition of releasing agents. The incubation was continued for 10 min at the respective temperature, except at 0°C, which was prolonged to 20 min. The reaction was stopped by dilution with 1:100 into 2 ml ice-cold Serenzen-A. Mean \pm S.E. of 3 expts.

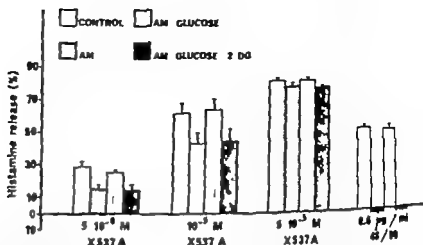


Fig. 5 The influence of antimycin, glucose and 2-deoxyglucose on histamine release induced by X537A and compound 48/80. After addition of X537A or compound 48/80 incubation in control for 10 min. Mean \pm S.E. of 4 expts.

tions of X537A, it was noted that Antimycin induced a slight, but significant release of histamine at low concentrations of X537A which caused submaximal release (Fig. 5). This inhibitory effect was counteracted by the simultaneous presence of glucose (2 mM) in the medium. The inhibitory effect of glucose was, in turn, abolished by the simultaneous presence of 10 mM 2-deoxyglucose (an inhibitor of glucose utilization). At higher concentrations of X537A ($5 \times 10^{-5} \text{ M}$) no influence of these metabolic inhibitors was observed. For comparison, histamine release induced by compound 48/80 was included, showing a complete inhibition by Antimycin, which was reversed by the presence of glucose. This effect of glucose was completely counteracted by the presence of 10 mM 2-deoxyglucose. The inhibitory effect of Antimycin together with 2-deoxyglucose on histamine release induced by X537A (10^{-5} M) from rat mast cells incubated in Sørensen-A was overcome by prolonged exposure of the cells with the releasing agent (4 expts. data not shown). The inhibitory action of metabolic inhibitors was notable at 10 and 20 min of exposure of the cells to X537A, but was absent at 30 and 45 min of exposure, in which cases the histamine release amounted to 70% or more. Thus, these agents decreased the rate of release induced by X537A without influencing the total release.

Discussion

The present results agree with those of Foreman *et al.* (1973) who found that only extracellular calcium did not inhibit histamine release induced by X537A. In fact, on the other hands extracellular calcium was found to depress the secretory action of X537A. Furthermore, in the absence of extracellular calcium pretreatment of the cells with either EDTA or A23187 which effectively diminished the response of the cells to compound 48/80 (Foreman and Ueda 1973; Diamant and Patkar 1975) did not, in our hands, influence the response to X537A. These results differ from those of Douglas and coworkers (Cochran and Douglas 1974; Kagayama and Douglas 1974) who found that EDTA-treated cells respond to X537A unless calcium was added to the cells. T

EDTA-treated cells is at present not clear. Our finding that there was no influence of EDTA or A23187 on histamine release by X537A does not favour the opinion that the mechanism of release depends on intracellular calcium. In our hands the presence of calcium depressed the secretory response of mast cells to X537A. The finding that it was evident when the cells were added to an incubation medium containing calcium as well as when they were preincubated with calcium before the addition of X537A indicates that inactivation of X537A by binding to calcium is not a likely explanation. This is further supported by the finding (unpublished observations) that barium, as a much greater affinity to X537A than calcium (Pressman 1973), was ineffective on the release induced by X537A. Since calcium was found to depress the rate of release by X537A, a more likely explanation for the inhibitory action of extracellular calcium might be a stabilizing effect on the plasma membrane of mast cells, which interferes with either uptake of X537A into the cells or with transport of monovalent cations across the plasma membrane. An inhibitory effect of calcium on the secretory action of X537A has also been reported for insulin secretion from pancreatic β -cells (Hellman

1973). Our present results indicate that monovalent cations are inherent in the mechanism by which X537A releases histamine from rat mast cells. In this respect our results agree with those concerning the release of vasopressin induced by X537A from neurohypophysis (Hosoi *et al.* 1976). Monovalent cations are known to exchange for histamine bound to its carrier matrix (Ulvén *et al.* 1970). Since they are carried by X537A over membranes (Pressman 1973), we suggest that they reach and release histamine from intracellular granules and the requirement of a calcium- and energy-dependent exocytotic process to establish a pore for exchange. The finding that the rate of histamine release was faster in Tris-sodium (150 mM) as compared with Tris-sodium (150 mM) is in agreement with observations concerning the influence of these ions on X537A-induced release of serotonin from blood platelets (Müller *et al.* 1976). This effect could be ascribed to a depolarization of the plasma membrane of intact rat mast cells by high potassium (Guzelin 1973), which could be mimicked by X537A, which by itself is known as a depolarizing agent (Cochrane and Johnston 1975). An additional possibility might be that potassium is more efficiently transported across membranes by X537A than sodium. An action of high potassium by itself on mast cells is suggested from the findings that the spontaneous release was higher in Tris-potassium (150 mM) than in Tris-sodium (140 mM) and amounted to $21.1 \pm 3.2\%$ and 0.7% respectively of the total histamine content after 10 min of incubation (mean \pm of 6 experiments).

Although the rate of release of histamine was found to be dependent on the concentration of X537A, it was generally slower than those observed with several agents which depend on chemical or metabolic energy for their secretory effects (compound 48/80, antigen, and A23187) (Bloom *et al.* 1967; Diamant *et al.* 1974; Diamant and Patkar 1975). The release of serotonin from blood platelets induced by X537A has also been noted to be slow as compared with that induced by other releasing agents (Müller *et al.* 1976).

The influence of metabolic inhibitors on the secretory action of X537A on mast cells had not been studied earlier by Foreman *et al.* (1973), who found that neither cyanide nor 2-deoxy-

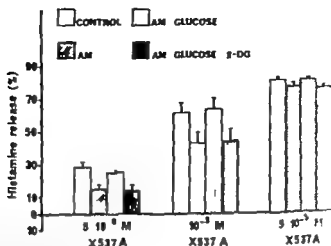


Fig. 5. The influence of antimycin, glucose and 2-deoxyglucose on histamine release induced by compound 48/80. After addition of X537A or compound 48/80. $\text{Mean} \pm \text{S.E.}$ (4 expts).

tions of X537A, it was noted that Antimycin induced a slight decrease in histamine release at low concentrations of X537A which caused submaximal release. This effect was counteracted by the simultaneous presence of glucose (2 mM). The inhibitory effect of glucose was, in turn, abolished by the simultaneous presence of 2-deoxyglucose (an inhibitor of glucose utilization). At high concentrations of X537A (5×10^{-5} M) no influence of these metabolic inhibitors was observed. The effect of histamine release induced by compound 48/80 was included significantly by Antimycin, which was reversed by the presence of glucose. The effect of Antimycin was completely counteracted by the presence of 10 mM 2-deoxyglucose. The effect of Antimycin together with 2-deoxyglucose on histamine release induced by compound 48/80 (10^{-5} M) from rat mast cells incubated in Sorensen-A was overcome by the presence of 10 mM glucose. The effect of the cells with the releasing agent (4 expts. data not shown). The effect of these metabolic inhibitors was notable at 10 and 20 min of exposure of the cells to the releasing agent. It was absent at 30 and 45 min of exposure, in which cases the histamine release was 70% or more. Thus, these agents decreased the rate of release induced by compound 48/80, influencing the total release.

Discussion

The present results agree with those of Foreman *et al.* (1973), who found that extracellular calcium did not inhibit histamine release induced by X537A. In our hands extracellular calcium was found to depress the secretory action of compound 48/80. Moreover, in the absence of extracellular calcium, pretreatment of the cells with A23187 or A23187 which effectively diminished the response of the cells to compound 48/80 (Foreman and Ueda 1973; Diamant and Patkar 1975), did not in our hands influence the action of X537A. These results differ from those of Douglas and coworkers (Douglas 1974; Kagayama and Douglas 1974), who found that EDTA-treated cells responded to X537A unless calcium was added to the cells. The reason for the dif-

EDTA-treated cells is at present not clear. Our finding that there was no influence of EDTA or A23187 on histamine release by X537A does not favour the opinion that the mechanism of release depends on intracellular calcium. In our hands the presence of calcium depressed the secretory response of mast cells to X537A. The finding that this was evident when the cells were added to an incubation medium containing calcium as well as when they were preincubated with calcium before the addition of X537A indicates that inactivation of X537A by binding to calcium is not a likely explanation. This is further supported by the finding (unpublished observations) that barium, which has a much greater affinity to X537A than calcium (Pressman 1973), was ineffective in depressing the release induced by X537A. Since calcium was found to depress the rate of release by X537A, a more likely explanation for the inhibitory action of extracellular calcium might be a stabilizing effect on the plasma membrane of mast cells, which could interfere with either uptake of X537A into the cells or with transport of monovalent cations across the plasma membrane. An inhibitory effect of calcium on the secretory action of insulin has also been reported for insulin secretion from pancreatic β -cells (Heflman

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Although the rate of release of histamine was found to be dependent on the concentration of X537A, it was generally slower than those observed with several agents which depend on metabolic energy for their secretory effects (compound 48/80, antigen, and A23187) (Bloom *et al.* 1967; Diamant *et al.* 1974; Diamant and Patkar 1975). The release of serotonin from blood platelets induced by X537A has also been noted to be slow as compared with that induced by other releasing agents (Müller *et al.* 1976). The influence of metabolic inhibitors on the secretory action of X537A on mast cells had been studied earlier by Foreman *et al.* (1970).

glucose influenced the action. The present investigation has in general confirmed results by using Antimycin and 2-deoxyglucose as metabolic inhibitors, although a depression of the rate of release was observed at suboptimal concentrations of X537A indicates that cellular ATP might affect the permeability properties of mast cell membrane by mechanisms which are at present unknown.

An additional finding which distinguishes the mechanism by which histamine is released by X537A from that induced by the ionophore A23187 and compound 48/80 was the observation that at 45°C X537A effectively released histamine, whereas the other two agents failed to do so. The action of X537A could also be distinguished from the simple hydrolytic action of decylamine since X537A did not release histamine at 0°C, whereas low temperatures did not abolish the releasing effect of decylamine.

It is concluded that the mechanism of histamine release induced by X537A differs from that exerted by calcium- and energy-dependent releasing agents on the one hand and from lytic agents on the other.

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Anticholeretic effect of substance P in anesthetized dogs

By

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Abstract

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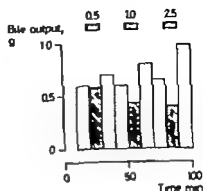
Nine anesthetized dogs were provided with acute common duct fistulas after excision of the pylorus. Synthetic substance P was administered as caval infusions in a dosage of 0.5-20 $\mu\text{g kg}^{-1} \text{ min}^{-1}$ for 10 min. The output of hepatic bile sodium and amylase decreased during infusion by 40-52 per cent at the highest doses. After termination of infusion all 3 parameters increased by 19-68 per cent above basal level. The biliary concentration of sodium was constant, while that of amylase increased during infusion. The responses were dose-related. The anticholeretic induced by substance P might be due to inhibition of the canalicular bile fraction, which presumably is mediated by active sodium transport independent of bile salt excretion.

Substance P (SP) (Euler and Gaddum 1931) is an undecapeptide present mainly in the central and peripheral nervous system and in the nervous structures and endocrine cells of the intestinal wall (Lembeck 1953, Pernow 1953, Amin, Crawford and Gaddum 1953, Nilsson *et al.* 1975, Pearce and Polak 1975). SP has an excitatory action on motoneurons of the spinal cord (Konishi and Otsuka 1974, Henry, Krnjević and Morris 1975) and induces vasodilatation (Pernow 1953, Pernow and Rosell 1975, Hallberg and Pernow 1975), contraction of smooth muscle (Euler and Gaddum 1931, Pernow 1953), and secretion from salivary glands (Lembeck *et al.* 1968) and pancreas (Starke *et al.* 1968). Starke *et al.* (1968) reported that crude SP extracted from intestine and brain caused contraction of the bladder in the dog. Hepatic bile flow was either not influenced or decreased slightly.

The aim of the present work was to study the mode of action of synthetic SP on biliary secretion. The total bile flow was chosen as the main parameter while sodium was chosen as a substance to represent biliary electrolyte excretion. In addition amylase was allowed to represent biliary enzyme output.

Experimental evidences for 3 different mechanisms of bile secretion have been reported (Wheeler 1968, Erlinger *et al.* 1970, Erlinger and Dhumeaux 1974). Two fractions of canalicular origin, one being bile acid dependent and the other bile acid independent, are suggested to be mediated by active transport.

Effects of caval infusions of SP on the output of bile. A sensitive experiment. Dosages: 0.5, 10 and 2.5 $\text{ng kg}^{-1} \text{min}^{-1}$



fraction is of duodenal-duodenal origin and stimulated mainly by secretin, which results in increased bile volume and a rise in bicarbonate concentration. Small single chain peptides, e.g. cholecystokinin, gastrin and vasoactive intestinal peptide, have been proposed to influence bile flow via the electrolyte (Hardman and Norman 1968, Wheeler 1968) or via the (Thulin and Hellgren 1976) excretion.

Material and Methods

Substance P (SP) was kindly supplied by Dr Karl Folkers, Institute for Biomedical Research, Austin, Texas. It was dissolved in sterile saline, passed through a bacterium filter (Kahl, Sweden) and kept frozen until used. The experiments were performed in 9 medium sized mongrel dogs. Anaesthesia was induced with pentobarbital, 25 $\text{mg kg}^{-1} \text{min}^{-1}$ and maintained at a steady state with repeated doses at regular intervals. No fluid or food was administered 15 min prior to SP infusions. The animals were endotracheally intubated and ventilated by an Engström respirator with $\text{N}_2\text{O} + \text{O}_2$. Mean arterial blood pressure was recorded electromechanically via a catheter in the femoral artery. Via a small subcostal incision, the cystic duct was ligated and common duct drainage created close to the liver hilus. Approximately 30 min after surgery the bile flow per 10 min was found to be constant. SP was infused in small amounts of saline into the inferior caval vein.

9 dogs were given a total of 23 caval infusions in dosages of 0.5–20 $\text{ng kg}^{-1} \text{min}^{-1}$ duration 10 min, and bile was sampled over 10 min periods. The parameters studied were total bile output and biliary content of sodium and electrolyte. The bile output was measured by weighing, the sodium content by flame spectrometry and the amylase content by starch hydrolyzing capacity (Phadebas, Pharmacia, Sweden). Statistical analysis of the data obtained was performed by the use of Student's *t*-test for correlated series. *p*-values less than 0.01 were considered statistically significant.

Results

1. Effect of SP on bile secretion

Caval infusions of SP in doses of 1.0 $\text{ng kg}^{-1} \text{min}^{-1}$ or higher induced a statistically significant anticholeretic effect. After termination of infusions there was a choleretic response, which however was not statistically significant at doses lower than 20 $\text{ng kg}^{-1} \text{min}^{-1}$. Thus, at an infusion rate of 20 $\text{ng kg}^{-1} \text{min}^{-1}$ the volume diminished from a mean control value of 1.3 g down to 0.6 g per 10 min. After termination of the infusions, the bile flow increased up to 1.7 g per 10 min (Table 1). The anticholeretic effect of SP occurred within 1 min after the beginning of the infusion while the post-infusion choleretic

TABLE I Effects of caval infusions of SP dosage $20 \text{ ng kg}^{-1} \times \text{min}$ on the output of bile and amylase p-values for change from control.

		Control	During infusion	After infusion
Bile g/10 min	\bar{x}	1.3	0.6	1.3
	Range	0.7-1.9	0.3-1.1	1-2.3
	n	7	7	7
	p <		0.001	0.01
Sodium mmol/10 min	\bar{x}	0.52	0.14	0.29
	Range	0.19-0.8	0.03-0.19	0-0.49
	n	2	2	2
	p <		NS	NS
Sodium, mmol/l	\bar{x}	170	175	178
	Range	167-177	171-179	165-181
	n	2	2	2
	p <		NS	NS
Amylase, $\mu \text{kat} \cdot 10^{-3}/10 \text{ min}$	\bar{x}	4.1	2.3	7.1
	Range	2.0-5.8	1.8-2.7	3.1-14.7
	n	7	7	7
	p <		0.01	NS
Amylase, $\mu \text{kat/l}$	\bar{x}	3.8	4.6	4.5
	Range	1.1-7.5	1.7-8.7	1.6-10.0
	n	7	7	7
	p <		0.001	NS

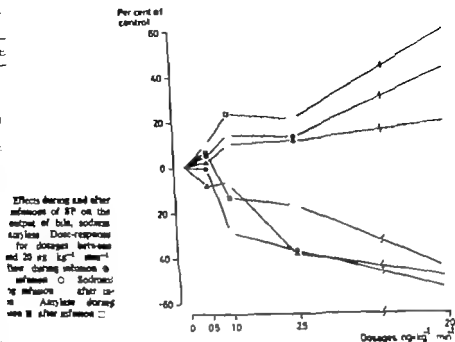
effect had a duration of about 10 min, whereafter the bile flow returned to the control level (Fig. 1). A clear dose-response relationship was demonstrated both for the anticholinergic and choleretic effect (Fig. 2).

B. Effect of SP on biliary sodium output

Caval infusions of SP in doses of $2.5 \text{ ng kg}^{-1} \text{ min}^{-1}$ or higher significantly decreased the total biliary output of sodium. At an infusion rate of $20 \text{ ng kg}^{-1} \text{ min}^{-1}$ only 2 animal analyses were performed because of the small bile volumes obtained. During an infusion rate of $2.5 \text{ ng kg}^{-1} \text{ min}^{-1}$ the total biliary output of sodium decreased from a mean control value of 0.19 mmol down to 0.12 mmol per 10 min. After infusion the sodium output returned to slightly above the control level (Table II). A dose-response correlation was found for the effects, both during and after infusion (Fig. 2). The concentration of sodium in bile ranged by less than 8 per cent in each animal and was thus constant before, during and after infusion (Table I, Table II).

C. Effect of SP on biliary amylase output

Caval infusions of SP decreased the total biliary output of amylase, the effect being significant only at the highest dose, $20 \text{ ng kg}^{-1} \text{ min}^{-1}$. However, already at a dosage of $10 \text{ ng kg}^{-1} \text{ min}^{-1}$ there was a significant increase in total amylase output after termination of the SP infusions (Table II, Fig. 2). Thus, the total biliary output of amylase decreased during SP infusions at the dosage of $20 \text{ ng kg}^{-1} \text{ min}^{-1}$ from a control value of 4.1



$2.3 \mu\text{kat } 10^{-3}$ per 10 min. After the infusion period the output was $7.1 \mu\text{kat } 10^{-3}$ per 10 min (Table I).

The concentration of amylase in bile rose from mean control value of 3.8 to 4.6 $\mu\text{kat } 10^{-3}$ during infusions of SP in doses of $20 \text{ ng kg}^{-1} \text{min}^{-1}$. The concentration of amylase in bile during the post-infusion period varied in the different animals compared to the controls and thus no significant changes were found (Table I).

Effect of SP on blood pressure

During oral infusions of SP at a rate of $2.5 \text{ ng kg}^{-1} \text{min}^{-1}$ and 10 min duration, there was an initial blood pressure drop from mean control value of 155 to 140 mmHg whereafter the pressure levelled off at 145 mmHg. Infusions of SP at a rate of $20 \text{ ng kg}^{-1} \text{min}^{-1}$ and 10 min duration induced a pressure drop of maximally 1 min duration from a mean control value of 145 to 100 mmHg. During the continued infusions the blood pressure stabilized at a mean of 120 mmHg.

Discussion

Motor effects of SP on the extrahepatic bile ducts have been studied previously in anesthetized dogs (Storke *et al.* 1968). The bile secretion was simultaneously studied after excision of the gallbladder. Crude SP-extracts of intestine and brain tissue were found to have no or only slightly diminishing effect on the bile flow. According to the authors, it could not be

TABLE II Effects of caudal infusions of SP dosage $0.5 \text{ ng} \times \text{kg}^{-1} \text{ min}^{-1}$ on the output of bile and amylase: p-values for change from control

		Control	During Infusion	After inf.
Bile g/10 min	<i>P</i>	11	0.7	13
	Range	0.7-1.7	0.4-1.2	0.9-1.3
	<i>n</i>	8	8	9
	<i>p</i> <		0.001	NS
Sodium, mmol/10 min	<i>P</i>	0.19	0.1	NS
	Range	0.13-0.28	0.06-0.23	0.12-0.20
	<i>n</i>	5	5	5
	<i>p</i> <		0.001	NS
Sodium, mmol/l	<i>P</i>	163	165	163
	Range	154-174	156-176	16-170
	<i>n</i>	5	5	5
	<i>p</i> <		NS	NS
Amylase $\mu\text{kat} \times 10^{-3}/10 \text{ min}$	<i>P</i>	3.7	3.0	4.4
	Range	1.7-5.7	1.0-6.1	2.0-12
	<i>n</i>	7	7	7
	<i>p</i> <		NS	0.01
Amylase $\mu\text{kat/l}$	<i>P</i>	3.7	4.3	4.1
	Range	1.9-5.7	1.1-6.6	1.9-11
	<i>n</i>	7	7	7
	<i>p</i> <		NS	NS

decided whether this effect was due to a reduction of hepatic blood flow or to an increase in the bile ducts.

In the present study SP was found to exert a biphasic action. Thus, during infusion of SP there was an anticholeretic response, while after termination of infusion a choleric response was recorded. It seems reasonable to believe that the essential effect of SP on the bile secretion is depressive. Anticholeresis is known to occur following infusion of toxic products (Wheeler 1968), but no physiologic substance has to our knowledge been demonstrated to depress bile flow.

The anticholeresis elicited by SP may be due to several factors. Some of these could be considered as indirect, such as a changed liver perfusion or bile duct contraction. Others could be considered as more direct such as enzymatic blocking of cellular synthesis or transport of bile. There is no reason to believe that the anticholeresis was due to the anaesthesia, since SP was administered during relatively brief intervals in a steady state of general anaesthesia. In fact pentothal sodium has been found to augment hepatic bile secretion (Erlinger and Dhumeaux 1974). Circulatory depressive effects of SP cannot explain the bile flow reduction since even extreme hypoperfusion evidently does not affect bile flow (Rytt et al. 1955; Fisher, Lee and Fedor 1958; Brauer 1965). Furthermore SP is known to increase both hepatic arterial and portal blood flow (Hallberg and Pernow 1975). An increase in the tone of the bile ducts as a cause of the anticholeresis could be ruled out by the experimental procedure used, since the common duct drainage was created close to the liver hilum. Furthermore, the bile canaliculi are known to be devoid of smooth muscle fibres.

The anticholeretic effects of SP might tentatively be elicited via the adrenergic or cholinergic

systems as a result of a neurotropic effect of SP. However it has not been possible to the hypotensive or smooth muscle stimulating effects of SP by anticholinergic or ergic blocking agents (Pernow 1953, Pernow and Rosell 1975). SP has been postulated for function in the sensory nervous system (Konishi and Otsuka 1974) and SP immuno-reactivity has been demonstrated in most tissues (Hökfelt *et al.* 1976). However no such immunofluorescence has been demonstrated in the liver (Hökfelt, personal notification). An anticholeretic effect of SP exerted via nervous influences thus seems likely.

Inhibition of synthesis is a possible explanation for the decrease in amylase output, however the instantaneous effect on the amylase output provoked by SP infusions is probably not in conformity of such a mechanism. Blocking of an active transport mechanism seems to remain as a plausible explanation of the anticholeretic effect of SP. A bile fraction of canalicular origin, dependent on active sodium transport, has been demonstrated. transport is mediated by Na⁺ K⁺-activated adenosine triphosphatase (Erlinger *et al.* 1974, Erlinger and Dhumeaux 1974). Inhibition of this fraction would result in a decreased biliary sodium output and a secondarily reduced bile volume during a maintained concentration of sodium in bile. This is in accordance with the findings in the present work: constant concentration of sodium in bile before, during and after stimulation with SP. Therefore, other small peptides with secretory effects such as cholecystokinin, vaso-intestinal peptide and secretin are considered to affect choleresis by such a mechanism (Lindan and Norman 1968, Wheeler 1968, Tholin and Hellgren 1976). SP is also known to augment the urinary output of sodium and water (Macfarlane, Mills and Ward 1974). However from the present experiments it is not possible to exclude that both sodium and water react secondarily to other factors such as changes in bile salt excretion. Studies on this problem are in progress.

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		Control	During infusion	After p
Bile g/10 min	\bar{x}	1.1	0.7	1.3
	Range	0.7-1.7	0.4-1.0	0.4-1.1
	n	8	8	8
	$p <$		0.001	NS
Sodium, mmol/10 min	\bar{x}	0.19	0.1	0.22
	Range	0.13-0.28	0.06-0.3	0.17-0.4
	n	5	5	5
	$p <$		0.001	NS
Sodium, mmol/l	\bar{x}	163	165	165
	Range	154-174	156-176	162-174
	n	5	5	5
	$p <$		NS	NS
Amylase $\mu \text{kat } 10^{-3}/10 \text{ min}$	\bar{x}	3.7	3.2	4.4
	Range	1.7-5.7	1.0-6.1	2.6-11
	n	7	7	7
	$p <$		NS	0.01
Amylase $\mu \text{kat/l}$	\bar{x}	3.7	4.3	4.1
	Range	1.9-5.7	1.1-6.6	1.9-17
	n	7	7	7
	$p <$		NS	NS

decided whether this effect was due to a reduction of hepatic blood flow or to a spasm in the bile ducts.

In the present study SP was found to exert a biphasic action. Thus, during infusion there was an anticholeretic response, while after termination of infusion a choleretic response was recorded. It seems reasonable to believe that the essential effect of SP on the bile flow is depressive. Anticholeresis is known to occur following infusion of toxic p -substances (Wheeler 1968) but no physiologic substance has to our knowledge been demonstrated to depress bile flow.

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Effects of the antidiuretic hormone, arginine vasotocin, theophylline, filipin and A23187 on cyclic AMP in isolated frog skin epithelium (*Rana temporaria*)

By

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Abstract

JOHNSON, A. H. and R. NIELSEN. *Effects of the antidiuretic hormone arginine vasotocin, theophylline, filipin and A23187 on cyclic AMP in isolated frog skin epithelium (Rana temporaria)*. Acta physiol. scand. 1978. 102. 281-289.

Methods for measuring cAMP in frog skin epithelium are developed. The epithelium is isolated after dispase-treatment. cAMP was extracted by boiling water and the extract is purified on dry Al_2O_3 . In response with time of the cAMP level after addition of arginine vasotocin (AVT) is studied. The hormone caused rapid increase in cAMP level with maximum after 3-5 min, hereafter the cAMP level declined. Incubation with AVT made the epithelium refractory to second dose of AVT. Such induction in the decline in cAMP level was caused by feedback mechanism and not by inactivation of the hormone. cAMP appeared evenly distributed in all cell-layers of the epithelium both before and after stimulation with AVT. Theophylline caused rapid increase in the cAMP level, which remained elevated for at least 15 min. Addition of the ionophore A23187 or of filipin had no effect on the cAMP level. However in the presence of theophylline, A23187 enhanced the cAMP level, whereas filipin had no effect. Therefore the mechanism of cAMP in the action of A23187 has to be considered.

It is well established that the addition of antidiuretic hormone (ADH) to the inside (the blood side) increases the active sodium transport and water permeability in various epithelia, among these frog skin and toad bladder. It is generally accepted that at least some of the effects observed after addition of ADH are mediated by cAMP. One of the tissues widely used for combined measurements of permeability changes and cAMP is the toad bladder and a number of reports have shown that ADH enhances the level of adenosine 3',5'-cyclic monophosphate (cAMP) in toad bladder (for references see Andreoli and Shafer 1976). The time course of cAMP level following addition of ADH has been investigated in toad bladder. Mendowitz et al. (1972) found the same cAMP levels 10 and 60 min after addition of ADH, and Floret et al. (1974) found that the cAMP level reached a steady state level 10 min after the addition of ADH and stayed there for at least 30 min, whereas Sapirstein and ¹ level reached a maximum after 3-6 min and then declined.

Concerning the effect of ADH on the isolated frog skin we know of only 2 reports. Shorderet Grosso and de Sousa (1975) found that incubation of isolated epithelia with ADH (oxytocin) plus 1 mM theophylline enhanced the cAMP level. 2) In their review ADH Jard and Bockaert (1975) referred to unpublished data according to which addition of ADH (oxytocin) in isolated epithelia resulted in a transient increase in the cAMP level. (The last two reports did not present methods or figures.)

The purpose of this study has been to develop a method for measuring cAMP in frog skin epithelium and to investigate the changes in cAMP level induced by ADH and theophylline. Furthermore it was investigated whether the activation of active sodium transport induced by filipin (a polyene antibiotic) and A23187 involved cAMP.

Materials and methods

Methods

The experiments were performed on male and female frogs (*Rana temporaria*). The frogs were kept permanently immersed in tap water at about 4°C.

It is well known, that there is a pronounced seasonal and biological variation in the different enzyme parameters. Concerning cAMP Hall *et al.* (1976) found a considerable difference between the cAMP levels in skin from winterfrogs and summerfrogs. The individual experimental series of the present communication were performed in different seasons, so it is to be expected that the cAMP levels obtained under otherwise equal conditions will vary from one series to the other. Therefore most of the experiments were performed on symmetrical halves, one half serving as control of the other.

The skins were mounted in perspex chambers (area 7 cm²) (Usung and Zerahn 1951) and bathed in oxygenated Ringer's solution (Na⁺ 115, K⁺ 5, Ca²⁺ 1, Mg²⁺ 1, Cl⁻ 116, CO₃²⁻ 2.5, SO₄²⁻ 1, PO₄³⁻ 1, glucose 10 mM, pH = 7.8). This solution was used for all incubations and referred to as "medium". The epithelia were isolated by collagenase treatment of the inside as described for *Rana lessonae* by Rajarmon *et al.* (1971) with the following modifications: 1) The skin was incubated without hydrostatic pressure and 2) at room temperature instead of 35°C, as the skin of *R. temporaria* did not survive at the higher temperature. After 1.5 h of incubation with collagenase the skin was washed twice, and when desired the skin was cut out of the chamber and placed on a glass plate and the intact epithelium was scraped off by means of a microscope slide. After the incubation (see below), cAMP was extracted by dropping the epithelium into 500 µl of distilled water containing 11-cAMP as recovery marker in a small test tube (pyrex glass, 75 µl) and placed in a boiling water bath. The tube was capped and 7 min later it was transferred to a ice-water bath. The content was homogenized 2 min with a rotating teflon pestle that fitted loosely. The test tube then homogenate was centrifuged for 10 min at 10 000 g in the same tube. 400 µl of the supernatant was placed on a microcolumn of 18 g dry Al₂O₃ on Pasteur pipette and eluted with 1 ml distilled water. The eluate was lyophilized, dissolved in buffer and cAMP was measured by a binding protein assay (Gierler *et al.* 1977) in duplicate at two dilutions.

The protein-precipitate which remained after the boiling and centrifugation was desalted by boiling 10 min in 1 ml 1 M NaOH and the protein was measured by a biuret method using bovine serum albumin (also dissolved in 1 M NaOH and boiled 10 min) as standard.

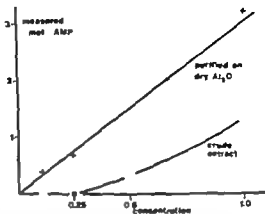
The effects of the various drugs on the epithelia were studied in two ways.

1) After incubation with collagenase the epithelium was isolated as described above and transferred to a test tube (polyethylen, 100-15 mm) containing 0.5 ml medium. The test tube was placed in a test tube rotator and equilibrated for 2 h after which the experiment was carried out under the desired conditions and cAMP was extracted as described above.

2) When exposure to drugs on only one side was desired (i.e. incubation with filipin and A23187) the procedure was a little different. After collagenase treatment the whole skin remained in the chamber, and incubation took place here. Thereafter the skin was cut out, the epithelium scraped off and cAMP was extracted.

When theophylline was included in the medium the sodium chloride content was reduced proportionally to keep the osmolarity constant as measured by freezing-point depression.

The short circuiting experiments were performed as described by Usung and Zerahn (1951).



An extract of frog skin epithelium, prepared as described in the text, was divided into two. One half remained untreated and the other was purified on dry Al_2O_3 and P was measured at 4 deletions in both.

Results of extraction and purification procedures

Extraction of CAMP on Al_2O_3 was originally described for separation of CAMP and ATP (the latter being fully adsorbed). This CAMP is not adsorbed) in an adenylate cyclase assay (Ramachandra 1971). It was found that the method was also sufficient for removing substances that interfered with the CAMP assay. It was performed on the crude water extract, as shown in Fig. 1. The extraction of CAMP by boiling, as described above, as compared to freeze-clamping in liquid nitrogen followed by extraction of CAMP in ice-cold trichloroacetic acid (TCA) and purification on dry Al_2O_3 (after repeated ether-extraction of the TCA-extract, lyophilization and redissolving in water). After treatment with organic solvents (AVT) alone we found much higher CAMP levels in the water extract than in the TCA extract (Table 1). This difference could have 2 explanations: either that the adenylate cyclase is inactivated by the heating or that the phosphodiesterase is not destroyed fast enough by TCA alone. That pretreatment with 30 mM theophylline eliminated the difference (Table 1) excluded the first explanation and the boiling method was chosen. It has been reported that the activity of phosphodiesterase (from beef heart) at 10°C is more than one half of the activity at 25°C (O'Dea, Haddock and Goldberg 1970). Therefore the reason for the consistently low freeze-clamping technique failed, might be that the pretreatment of TCA during thawing in ice-water was too slow due to the thickness of the epithelia which rolled up when they were lifted out of the TCA solution.

Materials

Theophylline (AVT), the neurohypophyseal hormone from amphibians (a gift from Szabo) is used in a final concentration of 40 $\mu\text{g/ml}$. Theophylline was obtained from Sigma. Flavin is a gift from the Upjohn Company) was used in a final concentration of 30 μM . ADP (a gift from Eli Lilly & Co) was used in a final concentration of 4 μM . Al_2O_3 (BDH and Merck) was in the neutral form.

Table 1 Extraction of CAMP from the epithelium with boiling water or TCA. Paired halves were incubated under the conditions given above. One half was freeze-clamped in liquid N_2 and CAMP extracted by neutral TCA. CAMP in the other half was extracted by boiling water. Values are pmol CAMP/mg protein \pm S.E.

Extraction	TCA	Boil. H_2O	Δ	
None				
3 min AVT	1.79 ± 0.04	1.75 ± 0.10	-0.04 ± 0.09	4
20 min theophylline	5.16 ± 0.71	11.37 ± 2.17	6.21 ± 1.41	6
3 min (theophylline AVT)	19.76 ± 2.65	19.31 ± 2.71	-0.46 ± 1.50	6

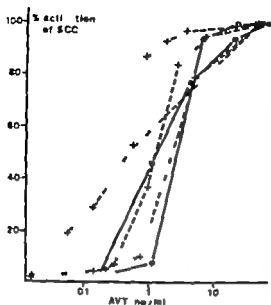


Fig. 2. Normalized (cumulative) response of SCC increasing AVT-concentration. Each expt. was conducted on paired halves, one remaining unstimulated as control to compensate for time-dependent fall of basal SCC. At start of expt. a. b. Percent activation of SCC as calculated as follows: % actual of SCC = $(a-b)/c \times 100$, where a = SCC of experimental half, b = SCC of control half, c = AVT-induced increase of SCC. +---+ 4 expts. with untreated skins, O—O, 2 separate expts. after preincubation with collagenase.

Collagenase (*C. histolyticum*) was obtained from Boehringer. Grade I was used in a final concentration of 135 mU/ml and grade II in a concentration of 45 mU/ml. Grade I was only used in half of the series with theophylline and fliquin the reason for replacing grade II with grade I in this series was that incubation with grade II was found to diminish the activation of SCC by fliquin considerably. Incubation with grade I did not (data not shown here).

Results

Effect of collagenase on AVT stimulation

It has been shown that the epithelia of *R. esculenta* preserve transporting capacity and responsiveness to stimulation by neurohypophyseal hormones after incubation with collagenase (Rajenison *et al.* 1972).

To test our preparation we compared the effect of AVT on the short-circuit current (SCC) of paired skin halves, one of which had been preincubated 1 h with collagenase. With collagenase the maximal increase of SCC following stimulation with AVT reached 61% above the resting level and with collagenase preincubation 49% (mean of 4 expts.).

We also tested the response to increasing doses of AVT (*i.e.* a cumulative dose-response). Curves from skins which had been preincubated 1 h with collagenase and from untreated skins were almost identical when per cent of maximal response was used as ordinate (Fig. 2).

These findings indicate that preincubation with collagenase (grade II) destroyed some of the AVT-receptors in the epithelium but had no effect on the remaining functional receptors.

Time-course of the response to AVT

The concentration of AVT (40 ng/ml) used in these expts. caused maximal stimulation of the SCC (Fig. 2). Paired halves were used for each measurement, one was kept unstimulated as control and the other incubated with AVT for the desired length of time. As it appeared that the levels of the controls varied fairly little in this series (1.92 ± 0.11 pmol cAMP/mg protein (mean \pm S.E., $n=15$)) and were substantially smaller than stimulated levels

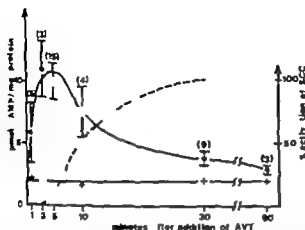


Fig. 3. Effect of AVT on the cAMP level in isolated frog skin epithelium. cAMP was extracted after the indicated time of incubation: ●—● cAMP level in the presence of AVT, mean \pm S.E., figures in parentheses number of experiments; +—+ cAMP in control epithelium. — — — typical increase in SCC after addition of AVT. Increase after 30 min of incubation = 100.

of halves were omitted in part of the measurements. After addition of AVT there was an increase in the cAMP level, which reached a maximum in 3–5 min and thereafter declined, reaching nearly the control level after 60 min (Fig. 3). The broken line in Fig. 3 is a typical response of SCC after addition of AVT.

The fall in cAMP after 5 min in spite of continuous exposure to AVT could be due to elution of the hormone. As seen from Table II epithelia which had been incubated in AVT for 60 min showed only a small increase in cAMP level 5 min after the addition of a second dose, and the cAMP level reached was far below that observed in epithelia only added to AVT for 5 min. This indicates that the observed decrease in cAMP level (Fig. 3) could not be explained by degradation of AVT.

Relationship between cAMP and epithelial protein content

Frog skin has a multilayered epithelium consisting of 3–7 cell-layers, and as we always used the same area of the epithelia, it seems reasonable to assume that the amounts of protein measured were proportional to the number of cell-layers. To examine how cAMP was

Table II. Effect of AVT on the cAMP level after 60 min preincubation with AVT. In the first series paired halves were incubated 65 min with AVT and a fresh dose of AVT was added to one half for the last 5 min. In the second series of paired halves one half was incubated 60 min with AVT and the other half remained untreated, after the 60 min period a fresh dose AVT was added to both halves for 5 min.

Incubation in AVT, min	paired cAMP/mg protein \pm S.E. Δ	
5	0.64 \pm 0.26	
5	1.44 \pm 0.40	0.61 \pm 0.17
5	2.85 \pm 0.35	
5	3.81 \pm 1.10	3.73 \pm 1.34
		4

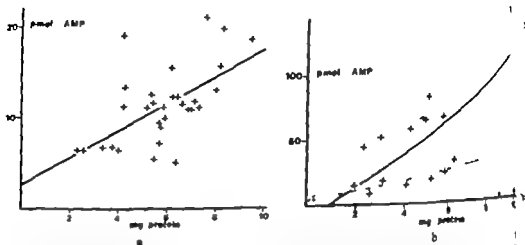


Fig. 4 a. Correlation between the amounts of cAMP and protein in unstimulated epithelia: $\text{pmol cAMP} = 1.51 \text{ mg protein} + 2.61$ $r = 0.59$ $p < 0.001$ Intercept of y-axis is not significantly different from 0.

Fig. 4 b. Correlation between the amounts of cAMP and protein in epithelia incubated 5 min with 10^{-6} M AVT: $\text{pmol cAMP} = 12.27 \text{ mg protein} - 10.79$ $r = 0.68$ $p < 0.01$ Intercept of y-axis is not significantly different from 0, $p = 0.15$.

distributed in the epithelia the total amounts of cAMP were plotted against the amount of protein. The points obtained from unstimulated epithelia showed a significant correlation (Fig. 4 a). Those from epithelia incubated 5 min with AVT also showed a significant correlation (Fig. 4 b). But looking at the latter figure it seemed as if the results fell into 2 groups, the regression lines of which are drawn as dashed lines. The deviations from zero of the slopes of both lines are significant (upper line $p < 0.01$ lower line $p < 0.05$) and so is the difference between the slopes ($p < 0.02$). The explanation remains obscure for the time being.

Time-course of response to theophylline

Theophylline is an inhibitor of the enzyme phosphodiesterase, which hydrolyses cAMP and it is known to mimic the effects of ADH (Orloff, Handler and Preston 1963) and cause an accumulation of cAMP in toad bladder (Handler *et al.* 1965).

In order to study how the cAMP level changed with time, paired halves were incubated for different periods with 10 mM theophylline. Three series were made. In the first series one half was incubated 15 min and the other half 5 min, in the other series incubation times were 5 and 15 min, and 15 and 45 min.

cAMP reached a new steady state level of 7.66 ± 0.33 pmol cAMP/mg protein (mean \pm S.E., $n = 8$) within 5 min and this level was maintained for at least 45 min. A few frogs responded considerably stronger than the major part and was omitted from the calculations above.

Effects of filipin and A23187

Both filipin and A23187 cause an activation of the SCC. Therefore it was investigated whether these drugs also had an effect on the cAMP level.

The addition of filipin or of A23187 alone to the inside had no effect on cAMP level (Table III). However, in the presence of 10 mM theophylline A23187 increased the cAMP level whereas filipin had no effect (Table IV).

- III Effect of flupar and A23187 on cAMP level. Paired halves were used. One half was kept as control while the other half received the ionophore for 10 min. Values are pmol cAMP/mg protein \pm S.E.

	Experiment	Control	Δ	
A23187 on inside	3.36 ± 0.27	2.90 ± 0.39	0.46 ± 0.32	5
flupar on inside	3.53 ± 0.18	3.17 ± 0.32	0.36 ± 0.30	5

Discussion

der *et al.* (1965) found that ADH increased the cAMP level in toad bladder and thereby sorted the hypothesis that the effects of ADH are mediated by cAMP. In the present communication it has been shown that the addition of the amphibian antidiuretic hormone, also caused an increase of the cAMP level in the frog skin epithelium (Fig. 3). The small increase in cAMP level following addition of AVT is contrasted by the considerable rise in SCC (Fig. 3). This finding is in accordance with the view that the physiological effects of ADH are not functions of cAMP concentration as such, but rather of the rate of phosphorylation of some membrane proteins, which again should be regulated by cAMP (Walton *et al.* 1975). Furthermore Fig. 3 shows that the increase in cAMP level was followed by a second phase with a fairly rapid decline. This raises the question how the response, observed during continuous exposure to AVT was brought about. From Table I it is seen that the response of the epithelium to a second dose of AVT was small after 60 min incubation with AVT. Likewise Huja and Hoog (1976), who measured SCC, found that the skin remained refractory to ADH for at least 2 h after the first dose had been washed off. Thus AVT appears to elicit negative feedback with a considerable delay time and after a short time explanation of this phenomenon seems possible. Either that the breakdown of cAMP by phosphodiesterase is enhanced or that the synthesis by adenylate cyclase is repressed. The second explanation is supported by the finding that the adenylate cyclase in pig kidney became desensitized during incubation with ADH (Roy Guillon and Ford 1976). Unlike the cAMP response to AVT the cAMP level following addition of 10 mM theophylline stayed elevated after a rapid increase. If the decline of the cAMP level in the second phase of stimulation with AVT is due to a lowered adenylate cyclase activity then the lack of decrease in cAMP level after stimulation with theophylline indicates that the feedback has no effect on the unstimulated adenylate cyclase, but only has an effect on the hormone

TABLE IV Effect of flupar and A23187 on cAMP level with 10 mM theophylline present. Paired halves were preincubated 15 min with 10 mM theophylline. One half received the ionophore for additionally 20 min, while the control half remained with no further additions. Values are pmol cAMP/mg protein \pm S.E.

	Experiment	Control	Δ	
10 μ M A23187 on inside	23.99 ± 3.50	14.93 ± 4.87	9.06 ± 1.48	4
10 μ M A23187 on outside	10.00 ± 2.30	6.16 ± 0.87	3.84 ± 1.94	4
20 μ M flupar on inside	13.54 ± 2.13	13.41 ± 2.20	0.13 ± 1.69	9

stimulated enzyme activity. The reason might be that a cAMP induced feedback mechanism uncouples a step between hormone binding and cAMP production. Or the feedback might be triggered from an element in the chain of events leading to stimulation of the adenylate cyclase.

Using compartmental analysis Morel and Leblanc (1973) claimed that sodium enters from the outside only into the outermost living cell-layer (first RCL) during normal circuit conditions. It has also been shown that ADH caused swelling merely of the first RCL (Voûte and Ussing 1970). This indicates that it is only the first RCL, which responds to ADH. This raises the question whether the hormonal stimulation of cAMP level actually occurs in the first RCL. If this were so the total amount of cAMP in stimulated epithelium should be rather independent of the number of cell-layers, while on the other hand the total amount of cAMP should be proportional to the number of cell-layers if they were all stimulated by AVT. From Fig. 4a it seems reasonable to conclude that cAMP in stimulated epithelia is distributed evenly in all cell layers of the epithelium. From Fig. 4b it seems likely that also the increase in the cAMP level elicited by AVT takes place in all layers, not only in the first RCL.

Both the polyene antibiotic filipin (Nielsen 1977a) and A23187 (Nielsen 1976) caused an increase in SCC when added to the inside of frog skins. This effect of filipin was abolished by preceding treatment with AVT (Nielsen 1977b). This was not due to a maximal stimulation of SCC by AVT since it was possible to activate the SCC further by diphenyltin chloride. Thus the activations of SCC by AVT and filipin may be mediated by the same mechanism. With the aim of elucidating a possible causal relationship between cAMP and SCC the effects of filipin and A23187 on the epithelial level of cAMP were investigated.

Neither filipin nor A23187 caused an increase in cAMP level by themselves (Table III). This indicates that the activation of SCC by filipin and A23187 does not involve cAMP and more generally that an elevation of the cAMP level is not a necessary prerequisite for activation of SCC. Then the possible common step in the action of AVT and filipin or A23187 must be at a point later than cAMP production.

With theophylline in the medium filipin did not cause an increase in cAMP level but A23187 did (Table IV). This may indicate that there is a difference—quantitative or qualitative—in their action, which is supported by the observation that the effect of filipin on SCC was abolished by preceding incubation with A23187 while preceding incubation with filipin did not abolish the stimulation of SCC by A23187 (Nielsen 1977b).

That an elevation of the cAMP level was only measured with theophylline present indicates that A23187 only caused a very small increase in the cAMP production. Localized increases in cAMP level may bring about physiological effects, but be undetectable unless the decomposition is reduced by theophylline. Still, the increase in cAMP level following A23187 in the presence of theophylline may be secondary to a primary effect of A23187 as ionophore, since divalent cations are known to affect the activities of adenylate cyclase (Bockaert, Roy and Jard 1972). Christophe *et al.* (1976) found that A23187 increased cAMP level in guinea pig pancreatic acinar cells, and ascribed this to an effect of the increased calcium level. Thus it may be that A23187 enhanced cellular concentration of the divalent cations (magnesium or calcium) and thereby caused an increase in cAMP level.

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The ribosome content of erythroid cells provides a good index of the degree of cellular maturation. In a transmission electron microscopy study Yamamoto and Iuchi (1976) show an increased electron-opacity of the cytoplasmic matrix due to accumulation of globin concomitant with the disappearance of the cytoplasmic organelles. There are present only few data concerning the kinetics of the maturation of erythroblasts into erythrocytes and the synthesis of hemoglobin during the maturation in the circulating blood of fish are available. In the present investigation an attempt has been made to establish data in salmon (*Salmo salar* L.) by developing a method based on the incorporation of ^{59}Fe into hemoglobin.

Material and methods

Material and preincubation. 3-4 month old salmon (*Salmo salar* L.) (mean weight \pm S.D. 215 g, range 1-9 g) supplied by the Fishery Board from the breeding plant and salmon hatchery in Årøy, Uppland, Sweden are used. The fish were preincubated for two weeks to resting, acclimated to tap water. The originally selected strain derives from an eider stockbreeding in some parts through with intensive selection: it is poor in oxygen and has fairly high but constant ion composition, 16-2 mmol/L Mg^{2+} , 0.3-0.6 mmol/L, and HCO_3^- about 5 mmol/L according to Höglund *et al.* (1972). The fish are fed daily with pellets (Salmon start-up pellets, Aqua Evras AB, Södertälje, Sweden). Experiments are performed during October-December. During the experimental period the water temperature dropped from about $+12$ to $+10^\circ\text{C}$.

Group of 3 year old salmon supplied by the Salmon Research Laboratory in Årøy, Norway are used for whole-body autoradiographic study. These fishes were kept under identical environmental conditions as 3-4 month old ones as described above.

Incubation of ^{59}Fe and preliminary whole-body autoradiography. The fishes were anesthetized with MS-222 (Benzocaine, Switzerland) and each specimen was given 1 μCi of ^{59}Fe (1-0.1 μg Fe as ferric citrate as source) sodium citrate and made isotonic with sodium chloride, Amersham England 1971 code 471429. The amount was injected into the peritoneal cavity in single dose.

For some specimens may easily cause bleeding, then handling small specimens. In order to ensure that the dose of ^{59}Fe administered was incorporated in the iron stores of the fish, i.e. into the liver, spleen, and kidney, and to get an estimation of the time factor for this incorporation, preliminary test was carried out with 2 year old salmon. Equivalent doses of ^{59}Fe were injected i.p. in one group and i.v. in other group. Three days later the fish were killed and whole body autoradiography as performed as described by Ullberg (1954, 1955).

Blood sampling and preparation of blood smears. Day 0 the fishes were given ^{59}Fe i.p., and blood samples taken 1, 4, 10, 15, 20, 41 and 52 days later. The fish were killed by blow on the skull and blood was drawn from the dorsal aorta as heparinized capillaries after transection of the aorta. The blood was from each fish were found on glass slides with 1% glucose aldehyde in isotonic solution (Fowler 1964). The isotonic solution contained per litre NaCl 6.00 g, KCl 0.20 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.10 g, NaHCO_3 0.20 g, CaCl_2 0.20 g, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.05 g, glucose 1.00 g, pH 7.3-7.4.

Autoradiography of blood smears. All blood samples fixed on glass slides were processed simultaneously in glass slides were dipped into mixture consisting of equal portions of sodium acetate (Hoford KJ) and 1% glycine in water. The preparations were exposed $0-4^\circ\text{C}$ for 15 days and then developed with Kodak D19. The RBC are finally stained in the solution of Giemsa (Azur-Eosin-Methylene blue) 0.05 M phosphate buffer at pH 7.2.

Microscopic examination and classification of RBC. Light microscope examination of the RBC was performed at the magnification of 720 times. The background grain counts were obtained by counting areas of the same size as the RBC. Ten such areas equally divided over the investigated part of the preparation were counted in each smear. A part of the background grain count derives from the ^{59}Fe bound to plasma proteins. As the background grain count was small compared to the grain count of the RBC the plasma proteins were neglected.

The RBC were classified according to the morphological criteria shown in Table I. The classification was based on the developmental series proposed by Cation (1951), Topf (1955), and Wessberg *et al.* (1972). Therefore the silver grains in the nuclear emulsion lying above the cell were counted and the loss and the

Maturation of circulating red blood cells in young Baltic salmon (*Salmo salar* L.)

By

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Abstract

HÄRDIG J. *Maturation of circulating red blood cells in young Baltic salmon (Salmo salar L.)* Acta physiol. scand. 1978. 102. 290-300.

The maturation of circulating red blood cells (RBC) in salmon (*Salmo salar* L.) has been studied. A differential RBC series of 6 classes based on morphological criteria was proposed. After a single dose of ^{59}Fe given to 5-6 months old salmon the incorporation of radioactivity in maturing RBC was followed by autoradiography on blood smears. The relative distribution of labelled RBC between the 6 classes of specimens taken 15, 20, 41 and 51 days after the injection of iron showed that the RBC matured in the proposed series. The projected area of the RBC increased by 100% during the maturation. The growth was caused by an elongation of the RBC. Thus the ratio between short and long axes of the RBC decreased with maturation and can be used as a measure of the degree of maturation. The uptake of active iron measured as grain count per RBC or per projected area of the RBC increased during the maturation in classes II-IV. Radioactive iron was not incorporated by mature RBC. The maturation time from class I to class VI was shorter than 41 days. RBC iron bound to non-haemoglobin proteins as a possible restriction in estimations of the haemoglobin concentrations of developing RBC is discussed.

Fish blood contains red blood cells (RBC) of different size and shape (Cotton 1951, Selman and Beams 1969, Yamamoto and Iuchi 1976). The RBC are released into the vascular system as comparatively small circular cells. As development proceeds the size increases and the RBC become more elongated or elliptical in form as described by Topf (1955). According to Topf the release occurs at the erythroblast stage (in the carp, *Cyprinus carpio* L.) when the synthesis of haemoglobin does not start until a stage designated proerythrocyte is reached. The whole cell as well as the nucleus of the proerythrocyte are elliptical in contrast to the more circular erythroblast with a rounded nucleus (Topf 1955). Furthermore, based on histological findings Cotton (1951) has distinguished young RBC from older ones in teleost fishes calling the round RBC reticulocyte. Other changes in the maturing RBC have been shown by Sekhon and Beams (1969) who found that polyribosomes and mitochondria were relatively numerous in the immature RBC, but decreased in number concomitantly with RBC maturation. These findings were verified by Yamamoto and Iuchi (1976) in their studies on erythrocytes in developing rainbow trout. They suggest



Figure 1. Photographs of organ sections (above) and autoradiographs (below) of two-summer old *Salmo trutta* injected three days earlier with ^{59}Fe : A, intraperitoneally; B, intravenously; 1 heart, 2 liver, 3 kidney.

The morphological changes of the RBC from classes I-VI are characterized mainly by increasing long axes and to some extent by decreasing short axes. This results in an increased projected area of the RBC from classes I-VI and a decreasing short/long axes ratio (Table II). The CV calculated for the mean values of ratio were lower in all classes as compared to corresponding CV for the mean values of projected area (Table II). Therefore it is judged that the ratio is preferable to the projected area as an index when morphometry and classification of developing RBC.

The frequency of labelled RBC in the proposed age classes of RBC is shown in Fig. 3. Labelled RBC per class are expressed as a percentage of all RBC (labelled + nonlabelled) in the four sampling occasions in Fig. 4. As all calculations are based on 4 and in one case (day 41) 3 specimens no statistical calculations except mean values were performed. It can be seen in Fig. 3 that the frequency of labelled RBC has a maximum in class III day 20, in class IV day 20, class V day 41 and class VI day 52. These successive changes with

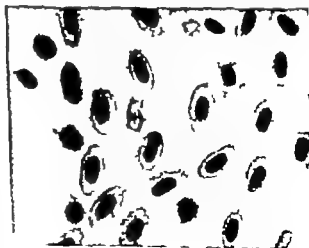


Figure 2. Typical autoradiograph of blood smear from a two-summer old *Salmo trutta* injected three days earlier with ^{59}Fe .

TABLE I Classification and morphological characteristics of circulating red blood cells (RBC) in rainbow trout. The cells are fixed with glutaric aldehyde in isotonic solution and stained with Giemsa solution.

Age class of RBC	Shape of cell projection	Appearance of nucleus		Subcellular cytoplasm colour
		Outline	Structure	
I	Circular	Circular	Dense	Light blue
II	Circular	Circular	Loose	Pale red
III	Slightly elliptical	Slightly elliptical	Loose	Red
IV	Slightly elliptical	Elliptical	Loose	Red
V	Elliptical	Elliptical	Loose	Red
VI	Elliptical	Elliptical	Dense	Red

short axes of the cell were measured by means of an ocular scale in the microscope. Sections from 15 fish were examined from day 15, 20, and 32 and 3 fishes from day 41. 20 to 30 cells in each class were counted. The ratio between the short and the long axes of individual RBC, the short/long axis index, was calculated as well as the projected area of each cell assuming a elliptical projection. On 30 RBC from each class the short and the long axes of the nucleus as well as the whole RBC were measured and the projected area of the nucleus, the whole cell, and the cytoplasm were calculated. All microscopic investigations were performed by one person.

Presentation of quantitative data and statistical treatment The 6 age classes of RBC are indicated by Roman numerals (I-VI). The quotient between the short axis and the long axis of the RBC is indicated as the short/long axis ratio.

Mean values of cellular morphometry given are followed by SE and coefficient of variation (CV). In regression lines were calculated using least squares and the coefficients obtained were tested using Student's T test.

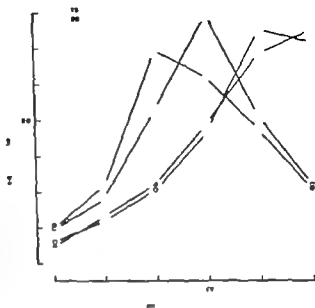
Results

The result of the whole-body autoradiography is exemplified in Fig. 1. In both cases the radionuclide appears in liver, spleen, kidney and in vascular organs. On the basis of these results ^{125}I injection was used in subsequent experiments.

Fig. 2 shows a typical part of an autoradiographic preparation of RBC used for the examination of radioactively labelled RBC. All over the emulsion there are tracts of background radiation. Generally the grain counts of labelled cells were 3-4 times the background. RBC were scored as labelled when their grain count was estimated to be twice the number of background grains. In a few fish the RBC showed extremely low incorporation, probably due to unsuccessful injection of radionuclide. Such specimens have been excluded.

The presentations in Table II and in Fig. 3-7 do not include counts from early parts of the tests, because there are no labelled cells in the autoradiographs from days 3 and 6 after the injections and after 10 days there are still few labelled RBC.

Results from morphological measurements of the RBC in the different classes are shown in Table II. The mean values of short and long axes have been used to present the "mean" cell in each class. The results from the measurements of the RBC nuclei are also presented in Table II. The ratio between the short and the long axes as well as the projected area of the RBC and the nucleus assuming them to be elliptical are given. The relative projected areas of whole RBC and of cytoplasm has been calculated from each class with reference to the mature RBC of class VI.



Since unlabelled as well as labelled RBC were found in all classes except in class III, day when the specimens were examined, the percentages of labelled RBC of all RBC in each class were counted as shown in Fig. 5. The highest percentages of labelled RBC were found in classes III-V. The low labelling per cent for class VI in all curves in Fig. 5 is due to the fact that this class consists of mature RBC and only a small amount of labelled RBC (compared with the mature pool) which have matured during the experimental period. Class V

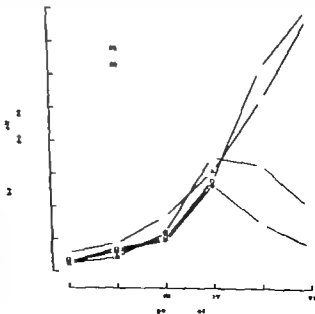
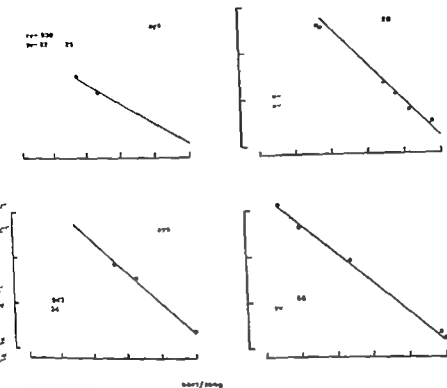


TABLE II Morphometry of young salmon RBC. The measures of cellular and nuclear short and long axes and the ratio of short/long axes as well as the projected area of the cell, nucleus and cytoplasm for 6 proposed age or maturation classes of RBC. Cell mean values, SE and CV (see text and methods pp 291) are based on all RBC examined, nucleus and cytoplasm values on least 30 measured cells in each class.

Age class of RBC		I	II	III	IV	V	VI
<i>Cell</i>							
Short axis (μm)	M	6.9	7.2	6.8	6.7	6.6	6.5
	SE	.28	.30	.16	.13	.13	.11
	CV	4.1	4.2	2.4	2.0	1.9	1.7
Long axis (μm)	M	7.7	9.1	10.0	10.9	12.1	13.1
	SE	.29	.23	.13	.12	.11	.10
	CV	3.7	2.6	1.3	1.1	.9	.8
Ratio	M	.90	.80	.69	.62	.55	.49
	SE	.01	.02	.01	.01	.01	.01
	CV	.89	.75	.38	.63	.53	.41
Area (μm^2)	M	41	51	54	57	63	68
	SE	.77	.69	.54	.40	.39	.33
	CV	1.9	1.3	1.0	.7	.6	.5
Relative RBC area		63	78	81	87	96	100
<i>Nucleus</i>							
Short axis (μm)	M	4.6	4.7	4.6	4.4	4.0	3.6
	SE	.09	.11	.06	.10	.08	.07
	CV	1.9	2.4	1.7	2.2	2.1	2.0
Long axis (μm)	M	4.8	5.4	5.9	5.9	6.3	6.7
	SE	.10	.11	.10	.09	.07	.07
	CV	2.1	2.1	1.7	1.5	1.1	1.0
Ratio	M	.96	.87	.79	.75	.63	.54
	SE	.02	.02	.01	.02	.01	.01
	CV	1.8	2.1	1.7	2.2	2.3	2.1
Area (μm^2)	M	18	20	21	20	19	18
	SE	.6	.8	.8	.6	.5	.5
	CV	3.5	4.1	3.6	3.1	2.4	2.3
Cytoplasm area (μm^2)	M	24	31	33	37	44	48
	SE	2.5	2.8	1.3	1.4	1.1	1.2
	CV	10.2	8.8	4.1	3.7	2.6	2.5
Relative cytoplasm area %		50	66	68	77	82	100

time in the distribution of labelled RBC from class III to class VI must be interpreted as a result of maturation of RBC from class I to class VI. Further support for this are the results of the calculations of per cent labelled RBC per class of all RBC (Fig. 4). It can be seen in Fig. 4 that only small differences seem to exist in these curves from classes I-IV day 20 to 52. Thus, after 20 days these classes are constant in their composition with respect to the quotient between labelled and unlabelled RBC. Accordingly the transit time (maturation time) for RBC from classes I-IV is shorter than 20 days. The level of labelled RBC in classes V and VI increases with time and days 41 and 52 have most likely the same distribution of labelled RBC. Thus the transit time for RBC from class I to class VI must be shorter than 41 days.



2. Incorporation of ^{59}Fe expressed as grain count per labelled RBC related to short/long axis ratio of fish independent of the classification in Table I. Regression lines are given for the decreasing phase of observation time.

The projected RBC cytoplasmic area is used as a measure of the RBC volume the highest concentration of radioactive iron in the cells is found at all the sampling occasions in class I. In this class the RBC has not achieved the final (mature) cell size. The radioactive iron grains per projected RBC cytoplasmic area) in RBC from class VI is almost the same for sampling days 20-52, with only a small but not significant increase.

The 6 classes represent a developmental series and the ratio between short and long axes of the RBC decreased from class I to class VI (Table II) and can be used as a measure of degree of RBC maturation. Therefore the grain count per RBC was plotted against the ratio between the short and long axes of the RBC (Fig. 7). The grain counts used are mean values calculated on RBC with equal ratios. As the grain counts are the same from 0.40-0.60 and then decrease with increasing ratio at days 15-41 the constants for the regression lines have been calculated for the decreasing parts of the curves. The constants for the regression lines are given in Fig. 7. The decreasing grain counts in classes III and IV from days 20-52 can be seen in Fig. 7 as decreasing slope for the regression lines.

Discussion

According to Fowler *et al.* (1966) and Orlic (1968) ^{59}Fe gives a good resolution in auto-

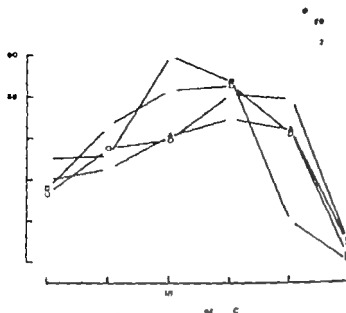


Fig. 5. Percentage of labeled RBC within each class I to VI (Table I) 15, 20, 41 and 52 days after the intravenous injection of a single dose of ^{55}Fe in each test fish.

day 15 consists of RBC which have taken up a significant part of their iron before the radioactive form became available to the cells and therefore shows a low grain count and many cells are not above the background count.

As the projected area of the RBC cytoplasm is increasing from class I to class VI (Table II) the number of grains per projected area of cytoplasm was calculated. From Fig. 6 it can be seen that the increase in grain counts (the uptake of iron) is not proportional to the increase of the cytoplasm area. The highest grain counts are found in class IV day 20. It can also be seen that in classes III and IV the grain counts decrease from day 20 to day 52. This is most likely a result of decreasing specific radioactivity of ^{55}Fe in the blood with time.

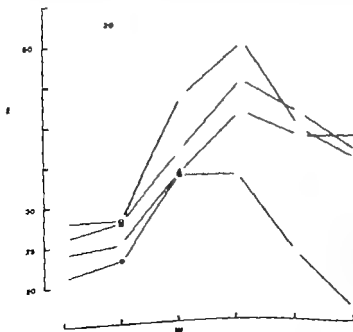


Fig. 6. Incorporation of ^{55}Fe in RBC belonging to each age class I to VI (Table I). The graphs represent the situation 15, 20, 41 and 52 days after the intravenous injection of a single dose of the radioisotope. The incorporation is expressed as number grain count per μm^2 of projected area (see Table II).

per RBC is achieved. This may explain the low detection of labelled RBC from the stages of RBC with low incorporation level (see Fig. 5). In later parts of the experiment the proportion of radioactive iron decreases and this will result in decreasing counts and a decreasing detection level of the RBC. This view is supported by the fact that the highest per cent labelled RBC is found on day 15 when the ratio between active and nonradioactive iron can be expected to be at maximum in the present study. The occurrence of ^{55}Fe in an early RBC as found in the present study does not necessarily mean that Hb-synthesis has started. Thus the iron detected by autoradiography may exist as Hb, b) other ferroproteins, c) ferritin or transferrin inside the cell, and d) transferrin bound to the outer surface of the cell membrane. In mature RBC Hb is by far the most ferroprotein. Hevery *et al.* (1964) showed that ^{55}Fe incorporated into RBC from the medium then 24 h (at 18°C) was bound to the Hb with low amounts in the non-Hb fraction. Walker and Fromm (1976) found that some iron accumulated by RBC in the young trout as temporarily stored as non-Hb iron. These authors also discussed the occurrence of non-Hb bound iron in the cytoplasmic iron stores as a result of over-loading of RBC.

Iron uptake by erythroid cells is not regulated by haem synthesis. Human developing erythrocytes have been shown to have an active uptake of iron from the iron-binding protein in the plasma and then return the excess (see e.g. Harris and Kellermeyer 1972). The iron uptake is likely regulated by the iron available to the cells (Harris and Kellermeyer 1972). Iron overloading of developing RBC may occur temporarily.

The blood cells in the present study did not show grain counts exceeding the background which supports the view that the iron incorporated into ferroproteins (cytochromes, peroxidases, and catalases) other than Hb can be neglected when the results in the present study are discussed.

Due to the fact that ^{55}Fe in the iron-binding proteins may be detected on the RBC membrane with higher efficiency than iron inside the cell and the possibility of non-Hb-bound iron and overloading, correlation between ^{55}Fe uptake and the increase of Hb concentration in the RBC is restricted. Thus the labelling (measured as grain counts) cannot be used as a quantitative measure of the RBC Hb-concentration. In young RBC with low Hb-concentration the discrepancy between Hb and iron is greater than in mature RBC. This will limit the estimations of the Hb-concentration in the different classes of RBC in the present study.

The decreasing number of grains per projected cytoplasm area in class IV from day 20 to day 52 (Fig. 6) and the decreasing slope of the regression lines for short/long axes ratio and grain counts (Fig. 7) could indicate overloading of the cells in class IV. This might also be a result of decreasing specific radioactivity in the plasma. It can be concluded that Hb is synthesized in the RBC during maturation along the projected series and it is not completed until the cells reach at least class IV short/long axes ratio 0.6.

No iron uptake was detected in RBC already mature at the time for the administration of the ^{55}Fe . This fact supports the theory that only the immature salmon RBC can take up iron as earlier has been found for mammalian RBC (cf. Harris and Kellermeyer 1972).

radiography presumably due to the comparatively low radiation energy of this isotope compared with ^{59}Fe . Furthermore, this technique was chosen in the present study as active iron incorporated in human erythrocyte haemoglobin according to Gibson *et al.* (1966) gives rise to a permanent activity during the whole lifetime of the red cells. Hovgaard (1964) and Weinberg *et al.* (1976) have shown that ^{59}Fe injected intraperitoneally in tench (*Tinca vulgaris*) and kissing gourami (*Helostoma temminckii*) appears in liver, spleen, kidney and RBC. Yu *et al.* (1971) report that ^{59}Fe incorporated into erythrocytes in kissing gourami (*Trichogaster trichopterus*) nine days after the administration appeared in liver and spleen. siderin in the same organs. According to the same authors this source of iron was exhausted during the erythropoiesis.

No reports have been found showing that fish erythrocytes are capable of cell division in the circulation. Thus it is assumed that RBC delivered to circulating blood only undergo maturational changes. Wintrobe (1974) presents a model of the production and kinetics of human leukocytes. In this model it is assumed that the cells move through the system in more or less orderly manner as if in a pipeline. In the present study class VI represents mature RBC and thus classes I-V can be interpreted as the maturation compartment. The change with time of the maximum of labelled RBC from classes III to VI (see Table 1) shown in Fig. 3 and the increase of labelled RBC in classes IV-VI with time presented in Fig. 4 must be interpreted as a flow of ageing RBC through the maturation compartment and classes I-V are interpreted as successive transformation stages in the RBC maturation compartment corresponding to the model of production and kinetics for human leukocytes presented by Wintrobe (*op. cit.*).

An estimation of the transit times for RBC through the maturation compartment can be obtained from the increase of labelled RBC in class VI (the mature RBC) from day 20 to day 34, which gives a result of 14 days, and similar estimations for the transit time to class V gives a result of 9 days. These transit times are only estimations since the sampling occasions have been chosen with too long intervals to permit exact calculations. No corrections have been made for differences in effectiveness in the labelling.

The autoradiographic technique used has certain limitations. Due to low radiation energy of ^{59}Fe , the grains observed in the emulsion mainly derive from disintegrations located in superficial parts of the RBC as radiation in the interior of the cells may be absorbed.

It was shown by Walker and Fromm (1976) that ^{59}Fe given intraperitoneally to rainbow trout (*Salmo gairdneri*) was adsorbed within 24 hours and equilibrium between the plasma ^{59}Fe pool and the tissue pool was attained 8 days after the i.p. injection. The half-life of the plasma iron in tench (*Tinca tinca*) has been determined to be 4.4 h by van Dijk (1964) and 4 h by Hevesy *et al.* (1964). According to this rapid turnover the specific radioactivity of the iron pool of the plasma and the pools of the tissues will be in equilibrium. The flooding of the fish body iron stores was done in the present study by parenteral administration of large doses of iron, the injected ^{59}Fe was mixed with iron already present. The iron pool will then persist for a rather long time compared to the situation with flooded iron stores. As only a part of the iron incorporated into the developing RBC is radioactive and only the iron in the part of the RBC which is close to the emulsion is detected with the autoradiographic technique used in the present study great variations in the detection

Insulin-stimulated bile formation in cats

By

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Abstract

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Effects of insulin on bile flow and composition were examined in fasting, chloralose-anesthetized cats. Insulin doses from 0.01 to 2.00 U/kg increased bile flow and biliary erythritol clearance. About any change in the difference between them, these insulin presumably had no effect on ductular fluid output. Continuous infusion of insulin (0.0 U/kg + 0.05 U/kg/min or 0.05 U/kg + 0.002 U/kg/min) increased biliary erythritol clearance by 22%. The increase was caused by rise in the bile acid-independent fraction of bile production and accompanied by parallel increase in the rates of biliary excretion of Na^+ and Cl^- . When insulin, 80 mg/kg, was injected intraperitoneally during insulin infusion the erythritol clearance, bile flow and the rates of biliary excretion of Na^+ and Cl^- were lowered towards but not to their pre-insulin levels. The effects of insulin on these parameters were unchanged after atropine or gastrostomy and were not affected by D-glucose as shown effect on bile production. The results indicate that administration of insulin affects bile formation by stimulating the active transport of sodium across the canalicular membrane.

Itz and Brooks (1963) first demonstrated the choleric effect of insulin in dogs. This effect has been confirmed by others and also demonstrated in guinea pigs and humans (see Jones 1976). However attempts to elucidate the mechanism of this choleric effect have given conflicting results. In guinea pigs it was recently found that insulin acted primarily by stimulating the secretion from the biliary ductal epithelium (Lundy Migliori and Jones 1977). Jones (1976) found that insulin stimulated the canalicular bile production in the dog with only minor effects on the ductular production of bile. In the present experiments the choleric effect of insulin has been examined in cats and the results show that insulin stimulates the bile acid-independent fraction of bile production apparently by stimulating the active transport of sodium across the canalicular membrane.

Methods

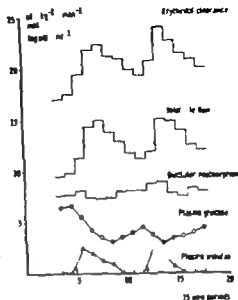
Young cats (weight 3.2 ± 0.1 (S.E.) kg), anesthetized with chloralose (50 mg/kg) and Nembutal (30 mg/kg) were used for the experiments. Catheters for infusion and blood sampling were placed in the femoral arteries and veins. Bile was collected from a catheter placed in the cholecyst after ligation of the

Thus cells belonging to the part of the developing RBC series which shows lower grain counts can be regarded as erythroid cells which synthesize Hb. The increase in Hb concentration cannot be correlated with increase in the detected iron. The Hb increase is most likely slower than the iron increase.

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The effects of injections of insulin 0.14 (first arrow) and 0.07 U/kg (second arrow) on flow and the arterial plasma concentrations of total insulin.

of insulin. The effects of a priming dose of insulin (0.8 U/kg) i. followed by a known infusion of insulin (0.05 U/kg/min) are listed in Table I and illustrated in Fig. 3. Administration of insulin resulted in an immediate rise in erythritol clearance which after 1 h stabilized at a level 22% above control flow for the rest of the experimental period. This was followed by a parallel increase in the Na⁺ and Cl⁻ excretion rates. There was a barely significant rise in the HCO₃⁻ excretion rate whereas the K⁺ and bile acids excretion rates remained constant. It also appears that the ductular reabsorption of fluid calculated as erythritol clearance minus total bile flow was unaffected by the infusion of insulin. It has been established that the maximum effects of insulin could be maintained for up to

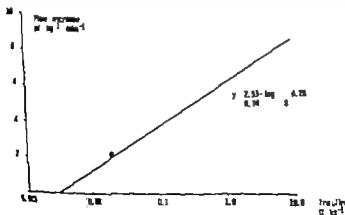


Fig. 3. The increase in total bile flow related to the amount of insulin injected.

cystic duct. To compensate for the abrupted enterohepatic circulation of bile acids (urocholester 8. kg/min) was given i.v. Mean arterial blood pressure was recorded using a condenser manometer (Schölander). Body temperature was maintained at 38.5°C by gentle heating.

Experimental design. In all expts. a period of 60 min was allowed for recovery from surgery. Arterial blood samples and bile were collected at 15-min intervals for 45 min. The following series were performed: a) 9 cats were given insulin (Insulin Leo® Neutral, Nordisk Insulinbioncern mark) in single doses i.v. ranging from 0.0015 U/kg to 2.0 U/kg, and the effects observed in 11 periods. b) 5 cats were given a priming dose of insulin of 0.8 U/kg followed by continuous i.v. insulin 0.05 U/kg/min and the effects observed in about 10 15-min periods. c) 5 cats were given as in series b. When the effect of insulin on bile flow had stabilized (after 75 min), ouabain was given into the portal vein in single doses beginning with 60 µg followed by 60 or 30 µg every 15 min until a level of about 80 µg/kg was reached. d) In 5 cats insulin was given as a priming dose of 0.05 U/kg followed by 0.002 U/kg/min and the effects followed for 10 15-min periods. e) In 4 cats ouabain alone was given partially in the same doses as in series c. f) The effect of insulin (0.8 U/kg followed by 0.05 U/kg) was also tested in 2 cats with acute total gastrectomy and in 2 cats in which atropine was given as a dose of 0.2 mg/kg i.v. followed by subcutaneous injection of 0.2 mg atropine 3 times during the maximal period. g) Finally the choleric effect of 2 Deo-γ-D-Glucose (Sigma) was tested in 3 cats were given 30–400 mg/kg i.v. as a single dose.

In all expts. plasma glucose and insulin concentration and bile flow and composition were determined. The biliary clearance of ¹⁴C-erythritol was determined by infusing 33 µCi/min i.v. together with 0.5 µmol erythritol (2 µmol/min) after priming doses of 3 µCi and 0.5 µmol, respectively.

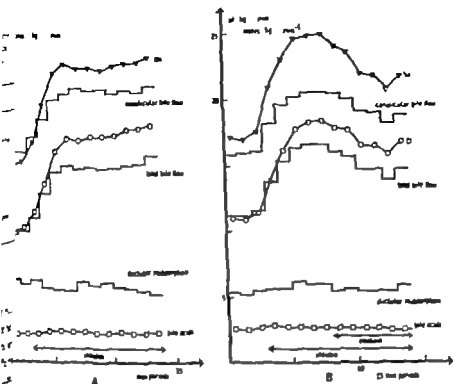
Analytical procedure. Bile flow was measured gravimetrically and the biliary concentrations of acids, inorganic electrolytes and plasma glucose were measured as previously described (Krupp and 1972). ¹⁴C-activity in bile and plasma specimens was measured in a Packard Tricarb liquid scintillation spectrometer and was corrected for quenching by means of an external standard (Krupp, Lee and Mueck 1976). Insulin was determined by a double-antibody technique using Wick chromatography (Thomson and Yde 1968).

Calculations. The biliary clearance of ¹⁴C-erythritol was calculated from the bile flow and the plasma ratio of ¹⁴C-erythritol. Assuming that this parameter reflects the canalicular bile production (Rozz and Bradley 1968) the net absorption/secretion in the bile ductules was calculated as the difference between erythritol clearance and bile flow. The biliary excretion of bile acids and salts was calculated as the product of bile flow and the biliary concentrations of the ions.

Statistical procedures. The effects of insulin on the flow and composition of the bile were tested by Student's *t* test for paired comparisons.

Results

Single injections of insulin. When insulin was injected i.v. in doses greater than 0.01 U/kg a rise in bile flow and a rise in ¹⁴C-erythritol clearance of similar magnitude was observed (Fig. 1). The increase in flow was manifest in the first sample after the injection and peak bile flow was reached 2–4 samples after injection, depending on the amount of insulin given. Also the time of return of flow to control values was dose-dependent. Insulin doses of 1–2 U/kg the plasma concentration of insulin rose above 500 µU/ml. A significant fall in plasma glucose concentration was observed as would be expected. Bile flow was often reached before nadir glucose concentrations and was independent of the initial plasma glucose level as well as the nadir plasma glucose concentration. When bile flow increase was related to the amount of insulin injected a correlation was found. Illustrated in Fig. 2. No obvious correlation between changes in bile flow and plasma insulin concentrations was observed. In these expts. the electrolyte excretion in bile was followed because of bile dead space interference.



5) The effect of insulin and insulin + ouabain on bile flow and composition. In part A insulin 0.8 U/kg/min was given as indicated. In part B the same dose of insulin was given but in the last 4 of the insulin infusion period ouabain was injected as indicated on the figure and described in the text.

erved in erythrocyte clearance or the fluid transport in bile ductuli. The composition of bile remained unchanged except for a rise in the biliary concentration of K⁺ and a corresponding rise in the excretion rate of this ion from 0.53 ± 0.03 to 0.79 ± 0.05 10^{-4} mol/kg/min (Table II).

11. Results from 4 experiments in which bile flow and the biliary excretion of Na⁺ and Cl⁻ were followed before and during injection of ouabain. The values are mean values \pm S.E. of the last 3 samples in the control period and the last 4 samples in the ouabain period. Ouabain values are not statistically different from control values.

	Control	Ouabain
total bile flow (10^{-4} kg ⁻¹ min ⁻¹)	11.0 ± 0.8	12.2 ± 0.5
erythrocyte clearance (10^{-4} kg ⁻¹ min ⁻¹)	14.7 ± 0.4	15.1 ± 0.9
excretion rate (10^{-4} mmol kg ⁻¹ min ⁻¹)	17.8 ± 0.1	18.6 ± 0.6
excretion rate (10^{-4} mmol kg ⁻¹ min ⁻¹)	12.2 ± 0.9	12.9 ± 0.6

TABLE 1 The effect of insulin (0.8 U/kg+0.05 U/kg/min) on bile flow and composition. The values are mean values \pm S.E. from samples 1-3 (control), samples 4-6 (insulin I), and sample 7 (insulin II) from 5 expts. in which only insulin was infused (A) and from 5 expts. in which insulin was injected during the last part of the insulin infusion period (B). Values from insulin I compared with values from control and values from insulin II with values from insulin I.

	Control	Insulin I	Insulin II
Total bile flow (μ l kg ⁻¹ min ⁻¹)	A 9.8 (1.4) B 11.3 (0.9)	13.0 (2.1) ** 16.4 (0.7) **	15.3 (2.2) ** 14.4 (0.7) * control
Canalicular bile flow (μ l kg ⁻¹ min ⁻¹)	A 16.2 (1.8) B 16.0 (1.4)	20.9 (2.4) 20.6 (1.2)	20.9 (2.4) ** 19.7 (0.7) * control
Na ⁺ excretion rate (10 ⁻³ moles kg ⁻¹ min ⁻¹)	A 15.1 (4.4) B 17.3 (1.3)	22.8 (3.2) 24.6 (1.3) *	23 (3.5) ** 21.4 (1.0) ** control
Cl ⁻ excretion rate (10 ⁻³ moles kg ⁻¹ min ⁻¹)	A 10.0 (2.1) B 11.2 (0.9)	17.3 (2.9) 19.2 (1.0)	18.1 (3.0) ** 16.5 (1.0) * control

ns = non-significant; * $0.01 < p < 0.05$; ** $0.005 < p < 0.01$; *** $0.001 < p < 0.005$.

eight 15-min periods a second series of expts. was carried out in which the effects of ouabain on insulin-stimulated bile flow was examined. In this series insulin was administered continuously as described above. When the effect of insulin on bile flow had become stable (after five 15 min periods) ouabain was injected in the portal vein in single doses for the rest of the experimental period as described under Methods. The results are listed in Table 2 and illustrated in Fig. 3. It appears that injection of ouabain caused a significant reduction in erythritol clearance and also reduced the Na⁺ and Cl⁻ excretion rates. Ouabain caused a slight non-significant rise in K⁺ excretion and a similar fall in HCO₃⁻ excretion rate, whereas the rate of excretion of bile acids remained constant. When steady state flow was achieved the plasma concentration of insulin was about 8-12 mU/ml.

The effect of insulin on bile flow and composition could also be produced by a lower dose of insulin of 0.05 U/kg followed by infusion of 0.002 U/kg/min. In these expts. the erythritol clearance increased from 14.5 ± 1.0 to 18.6 ± 1.1 μ l/kg/min, and the rates of excretion of Na⁺ and Cl⁻ increased from 16.5 ± 2.3 to 22.1 ± 2.3 and 10.8 ± 2.1 to 16.0 ± 2.3 10^{-3} mol/kg/min, respectively. Insulin caused a slight, but significant rise in HCO₃⁻ excretion rate from 2.5 ± 0.2 to 3.5 ± 0.3 10^{-3} mol/kg/min and similarly a fall in K⁺ excretion rate from 0.5 ± 0.02 to 0.4 ± 0.02 10^{-3} mol/kg/min was observed. The rates of excretion of bile acids was not changed. Once steady state flow was achieved the mean plasma insulin concentration was 105 ± 11 μ U/ml.

The plasma concentration of insulin in the control periods of the injection and the infusion expts. have been pooled together. In the first plasma sample following the operation procedure insulin concentration varied considerably between 3-168 μ U/ml with a mean value of 39 ± 11 μ U/ml. 45 min later just before administration of insulin, the variation had declined to 2-72 μ U/ml with a mean value of 41 ± 5 μ U/ml.

Injection of ouabain. In 4 cats the effects of ouabain alone on bile flow and composition was tested. Ouabain was given as single injections in the portal vein as described above and in similar doses. Ouabain did not affect total bile flow rate or was any change

insulin-induced rise in erythritol clearance accompanied by a marked, parallel increase and Cl^- excretion rates and a small increase in HCO_3^- excretion indicate that insulin stimulates a Na^+/K^+ -ATPase in bile canaliculi. If this was the case, ouabain would be able to abolish these effects of insulin. The limited effect of ouabain neither proves nor disproves the validity of this concept. The fact that we found only partial depression of insulin-induced increments in bile flow and Na^+ excretion may have been due to post doses of ouabain; this could also explain the lack of effect of ouabain on bile flow in the control expts. However, attempts to increase ouabain doses beyond 80 $\mu\text{g}/\text{kg}$ invariably resulted in sudden cardiac failure.

Whether insulin influences bile secretion directly or indirectly cannot be answered conclusively but several ways of action may be excluded. Neither total gastrectomy which includes truncal vagotomy nor injection of atropine interfered with insulin-induced choleresis in the cat. The vagus is thus excluded as a possible mediator of the insulin effect. The lack of effect of gastrectomy on the choleric action of insulin also excludes gastrin as a mediator of insulin effect. The constancy of the ductular fluid transport, as well as the lack of effect of biliary excretion of HCO_3^- during insulin administration makes it very unlikely that secretin should be responsible for the effect of insulin in the cat. Glucagon has been suggested as a possible mediator of the insulin effect, but previous expts. on cats (Krurup and Larsen 1974) demonstrated that glucagon, even when infused in high doses, gives a characteristic delayed choleric response. cAMP, a second messenger coupled with the action of glucagon and its dibutyryl derivative were without effect on bile flow (Krurup *et al.* 1975). Prostaglandins E_1 and E_2 are potent choleric agents in the cat (Krurup *et al.* 1976), but exhibit a secretion-like effect without influencing canalicular bile production. It is well known that hypoglycemia is accompanied by increased production of catecholamines but epinephrine and norepinephrine do not influence bile flow in cats (Krurup 1973).

Hypoglycemia, as such, has been suggested to evoke an increase in bile flow but in the present study single injections of insulin there was no correlation between insulin-induced bile flow and plasma glucose concentration. In contrast, the results from the infusion expts. show that bile flow and plasma glucose concentration are closely related. These results therefore suggest that hypoglycemia could be directly responsible for the increase in bile flow. However, in agreement with the expts. of Konturek, Kleja-Fyda and Moczurad (1967) in cats, but contrary to findings in dogs (Jones *et al.* 1970, Geisler, Jones and Hall 1970, and Jarley and Jones 1972), the cytotoxic agent, 2 Deoxy D-Glucose, did not effect bile flow which seems to speak against a direct effect of hypoglycemia.

The question then arises whether the choleric effect might be caused directly by insulin. The main condition for direct action of insulin seems to be fulfilled, namely that insulin would come into contact with the ATPase sites in the bile canaliculi membrane. It is well established that insulin is inactivated by the liver but according to the work of Lopez-Fernandez and Goffi (1967) and Daniel and Henderson (1970) insulin can pass the liver cell apparently undamaged in a variety of species including the cat, and insulin has been found in bile in the same concentration as found in portal venous blood. It was further calculated that the time required for insulin to pass through the liver cell to the bile was about 17 min. Based on this information it would be expected that rather than being

Gastrectomy and administration of atropine or 2 Deoxy D-Glucose For further definition of the mechanism of action of insulin on bile flow a series of experiments was carried out in which the effect of acute gastrectomy or administration of atropine (see methods) were tested. Two cats were gastrectomized and following the usual recovery and control period insulin was given as a single dose of 0.8 U/kg supplemented by 0.05 U/kg/hr. Bile flow and composition in the control period appeared to be unaffected by gastrectomy. The infusion of insulin was accompanied by the choleric response normally observed in other cats. It was found that administration of atropine in doses which are supposed to block all vagal reflexes, did not alter the choleric effect of insulin. Finally 3 cats were given 2 Deoxy D-Glucose as single doses ranging from 50 to 400 mg/kg. It appeared that this cytogluco-penic agent did not alter bile flow or the electrolyte composition of bile in any way.

Discussion

The present experiments have shown that injection of insulin stimulates bile flow also in the cat. The effect of graded doses of insulin on bile flow is in accordance with the report of Jones, Geist and Hall (1970) on conscious dogs and an increase in flow rate of about 5 l/kg/min at an insulin dose of 1-2 U/kg is compatible with the results of these and other experiments (e.g. Geist and Jones 1971 a, b, Gourlay and Jones 1972). This indicates that the experimental procedures employed including anaesthesia, is without major importance for this effect of insulin. According to Jones *et al* (1970) the effect of insulin reaches a maximum at a dose of 1 U/kg. This saturation phenomenon is also observed in the experiments with continuous infusion of insulin in which a 20 fold increase in the amount of insulin given, did not cause a corresponding rise in plasma insulin concentration from about 100 μ U/ml to 2-3 mU/ml. This was without significant influence on the increase in bile flow. Thus the effect of insulin on bile flow is only dose-dependent within certain narrow limits. This may explain why no correlation between the rise in bile flow and the plasma insulin and glucose concentrations was found in the single injection experiments.

The experiments with continuous infusion of insulin reveal that the effect of insulin on bile flow can be maintained for hours. Judged from erythritol clearance, insulin increased the canalicular production of bile flow while the calculated ductular net fluid transport appeared to be constant. This is in accordance with the findings of Jones (1976) on dogs but in contrast to the results of Lundy *et al* (1973) with guinea pigs, where insulin stimulated the ductular portion of bile production. Jones (1976) found a small increase in the biliary excretion rate of bile acids but in our experiments the excretion rate of bile acids was unaffected. This means that insulin infusion selectively stimulates the bile acid-independent fraction of canalicular bile production. This portion of bile production is claimed to be dependent on the active transport of inorganic ions and the inhibitory effect of ouabain on this bile production (Erlinger *et al* 1970, Boyer 1971) suggests that the active transport is due to a Na⁺-K⁺ ATPase. This is supported by the existence of Na⁺-K⁺ ATPase activity in bile canalicular enriched plasma membranes from rat liver (see Reichen and Paumgartner 1977), and experiments in progress in our laboratory have also shown Na⁺-K⁺ ATPase activity in isolated bile canaliculi from the cat.

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correlated to arterial plasma insulin concentrations, bile flow should be correlated to insulin concentrations in bile or portal venous blood. However these measurements have not been made.

It would be of interest to know whether insulin affects the activity of the Na-K-ATPase system from the bile canaliculi "in vitro" but no information is available on this point. However in expts. with toad bladder the results indicated that insulin stimulates the active sodium transport across the membrane by unmasking latent pump sites and by stimulation of formation of new pump sites (see Wiesmann, Sinha and Klahr 1977). If this mode of action is applied to the present results it may explain the difference in sensitivity of bile production to ouabain in the control and insulin expts.

In conclusion, the present expts. have demonstrated that in the cat administration of insulin stimulates the bile acid-independent fraction of canalicular bile production and probably by stimulating the active transport of sodium across the canalicular membrane. Whether or not this effect is caused by a direct action of insulin remains to be shown. In the results suggest that this may be the case.

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The results indicated that energy flux decreased somewhat during the first 30 min, following administration of amphetamine in a dose of 5 mg kg⁻¹ but that there was a post increase by 25–30%. Furthermore, two groups of workers (Naborski and 1975 King *et al* 1975) have observed that amphetamine administration leads to an increase in tissue concentrations of malate and, following the suggestion of Goldberg *et al.* (1975), this was assumed to reflect an increased metabolic rate. Recently we reported in preliminary communications that amphetamine sulphate in doses (5–15 mg kg⁻¹) induced a marked increase in CBF and CMR_{O₂} in paralyzed rats (Carbon *et al* 1975, Bernstein *et al* 1976). Under some circumstances, the increase in CBF was so pronounced that it became a matter of speculation whether the method used was of sufficient accuracy to allow reliable derivation of CMR_{O₂}. We therefore undertook a modification of the technique, designed to increase accuracy of the estimation of CBF and CMR_{O₂} in high flow situations (Bernstein *et al* 1978). In the present experiments, we have repeated and extended the previous studies on circulatory and metabolic effects of amphetamine in paralyzed and artificially ventilated rats, added measurements of regional CBF (rCBF) and performed extensive measurements of tissue metabolites. It will be shown that amphetamine markedly increases CBF especially in frontoparietal cortical regions, but that the effects on CMR_{O₂} are less than previously reported. Apart from causing glycogenolysis, amphetamine has small effects on tissue metabolites but, after large doses and long exposure periods, significant changes are observed in some glycolytic metabolites and citric acid cycle intermediates.

Methods

Experiments were performed on male Wistar rats, weighing 305–395 g. Animals were housed in pairs of 4 in plastic cages, and had free access to pellet food and water. Experiments were performed between 08.00 and 18.00 h.

Anaesthesia and sampling techniques

Unconscious animals were used for studies of classical behaviour following intraperitoneal injection of d,l-amphetamine sulphate in doses of 5 or 15 mg kg⁻¹. Other animals were provided with tail artery catheters under light anaesthesia (see Poulsen and Sjöberg 1967, Levin *et al* 1973). About 2 h later, when the animals had recovered from the anaesthesia, arterial samples were collected before and after i.p. injection of amphetamine (5 mg kg⁻¹) for measurements of P₅₀, P₅₀, and pH.

All other experiments were conducted on animals that were anaesthetized with 2–3% halothane (operative preparation) and then maintained on 70% N₂O and 30% O₂ without any halothane. Intubation was achieved with a cuffed endotracheal tube (1 mm i.d.), and artificial ventilation was adjusted to give arterial CO₂ tensions of 35–40 mmHg. Body temperature was adjusted close to 37°C.

In animals used for studies of CBF and CMR_{O₂}, two femoral arteries and one femoral vein were cannulated, and the posterior part of the superior sagittal sinus was exposed for sampling of cerebral venous blood. When rCBF was studied, no burr holes were placed, and one of the arterial catheters was cut to a length of about 2 cm (to minimize bleeding). Preparations for studies of tissue metabolites involved placing a sterile incision over the intact skull bone for later use in brain freezing according to Poulsen *et al.* (1974). In many of these animals, the atlantooccipital membrane was exposed for sampling of diencephalic CSF in sharp glass pipette.

Methods for CBF and CMR_{O₂}

Cerebral blood flow (CBF) was measured in two series of animals (A and B). Series A was studied in the fall of 1973 and spring of 1976 and the results have been summarized in two preliminary communications.

Circulatory and metabolic effects in the brain induced by amphetamine sulphate

By

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Abstract

BERNTMAN, L., CARLSSON, C., HÄGERDAL, M. and Sjöström, B. K. Circulatory and metabolic effects in the brain induced by amphetamine sulphate. *Acta physiol. scand.* 1978. 102. 310-323

Cerebral circulatory and metabolic effects of amphetamine sulphate ($0.25\text{--}30\text{ mg kg}^{-1}$ or $5\text{--}30\text{ mg L.p.}$) were studied in anesthetized, paralyzed and artificially ventilated rats. Cerebral blood flow was measured with a modification of the Kety and Schmidt (1948) technique, and oxygen consumption (CMRO_2) was calculated from CBF and arteriovenous differences in oxygen content. Regional CBF evaluated from the uptake of ^{14}C -ethanol. Cortical metabolites were analysed following freezing *in situ*. Amphetamine administration gave rise to a marked increase in CBF that was doubled for 0.25 mg kg^{-1} and increased 4-fold following 15 mg kg^{-1} . However, such excessive increases in flow confined to frontoparietal cortical regions, while other cortical or subcortical areas showed none or hyperemia. The increase in CBF was unrelated to changes in arterial Pco_2 , blood pressure, or tissue content. CMRO_2 increased by 30% to 95% depending on dose and rat strain used. At all doses amphetamine gave rise to glycogenolysis in cerebral cortex but in animals studied within the first hour after 5 mg kg^{-1} or less, the only other changes were increases in glucose-6-phosphate and α -ketoglutarate concentrations. When the dose was increased to 15 mg kg^{-1} there were moderate increases in lactate concentration and lactate/pyruvate ratio. Sixty min after 5 mg kg^{-1} there were increases in tissue concentrations of pyruvate, citric acid cycle intermediates and alanine, as well.

It has been well established that administration of amphetamine to rats and mice increases locomotor activity and induces a stereotyped behaviour (see symposium edited by Garattini 1970). Biochemical and pharmacological studies indicate that the effect is due to release of catecholamines from central monoaminergic neurons (e.g. Carlsson 1970). Several studies have shown that amphetamine leads to glycogenolysis in the brain (Estler and Ammon 1967, Hutchins and Rogers 1970, 1971, Rogers and Hutchins 1973, Nahorski and Rogers 1973; however see also King *et al.* 1975) probably by mechanism involving conversion of phosphorylase *b* to *a* (Nahorski and Rogers 1974). There is indirect evidence that amphetamine increases metabolic rate in the brain. Nahorski and Rogers (1973) applied the "closed system" method of Lowry *et al.* (1962) to estimate

ox, lactate and pyruvate were measured in arterial blood and CBF as well. Intracellular glucose concentrations were calculated by assuming that blood and extracellular fluid occupy 3 and 15 % respectively of tissue volume, and that CBF and extracellular glucose concentrations are equal.

III
 All significance of differences in means are determined by comparing control values with experimental values, using Student's *t*-test.

NS $p > 0.01$ ** $p < 0.001$ ***

Results

Results will be presented under the following headings: (1) behavioural effects, (2) effects on physiological variables, (3) glucose distribution, (4) tissue metabolites, and (5) CBF and CMRO₂. Tissue metabolites (and glucose distribution) were only measured in series A while the other variables were studied in both series A and B (see Methods).

Behavioural effects

Anesthetized animals of both series were injected *l.p.* with amphetamine in doses of 5 or 10 mg kg⁻¹ and their behaviour was observed for at least 60 min. All animals showed signs in behaviour previously reported by others, *i.e.* increased mobility, piloerection, salivation, salivation and stereotyped gnawing and rearing. The symptoms were usually observable within 5–10 min, the stereotyped behaviour being most marked following 15 mg kg⁻¹.

Effects on physiological variables

Five animals of series A and 2 of series B were provided with tail artery catheters under sodium anaesthesia. About 2 h later when the animals had fully recovered, they were injected *l.p.* with 5 mg kg⁻¹ of amphetamine. Arterial samples were collected every 15 min for measurements of P_{aO_2} , P_{aCO_2} and pH. There were no consistent variations in P_{aO_2} . Changes in P_{aCO_2} and pH are shown in Fig. 2. In animals of series A, mean P_{aCO_2} fell to about 27 mmHg within the first 15 min, and values remained below 30 mmHg during the 60 min observation period. Arterial pH varied in accordance with the fall in P_{aCO_2} . The two animals of series B showed a less pronounced hyperventilation, values remaining between 30 and 35 mmHg during the 45 min observation period.

Previous results have shown that administration of amphetamine to spontaneously breathing animals leads to hyperthermia (see Lewander 1971; Nahoraki and Rogers 1976). Since both hyperthermia and hypocapnia can, by themselves, induce changes in cerebral blood flow and metabolism, the ventilation of the present anesthetized animals was adjusted to maintain P_{aCO_2} at 35–40 mmHg, and body temperature was kept close to 37°C (since 100% O₂ was given, arterial P_{aO_2} was above 100 mmHg in all animals). Our main concern was therefore changes in blood pressure and pH as well as in lactate and pyruvate concentrations.

Rats under 70% N₂O usually have blood pressures around 150 mmHg. Following *l.p.* injection of amphetamine in doses of 0.25–25 mg kg⁻¹ there was invariably an increase in blood pressure of 10–25 mmHg, an example of which is given in Fig. 3. However, this in-

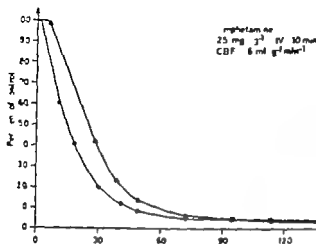


Fig. 1 Representative HbO_2 down curves obtained after intravenous injection of amphetamine (25 mg/kg). Circles arterial samples and triangles venous. Time in sec.

(Carlsson *et al.* 1975, Bernthman *et al.* 1976). Series B was studied after July 1976. In the summer of 1976 a new supplier of animals bred a new strain of Wistar SPF rats. In both series, the basic methodology was the same (Kety and Schmidt 1948) and involved saturation of animals with ^{15}O -gas, and repeated measurement of ^{15}O -gas activity in arterial and cerebral venous blood during desaturation (Eklöf *et al.* 1973, Norder and Sjöström 1974). However in series A a 5 sec lag period was allowed, following start of desaturation, before the first blood samples were collected, and it was seldom possible to collect more than 3 blood samples in the first minute of desaturation. This method has previously been used to measure CBF (and CMR) in two other high flow situations, *viz.* hypercapnia and hypoxia (Eklöf *et al.* 1973, Jönasson and Sjöström 1974). Since accuracy at these high flow rates was considered unsatisfactory the method was further modified with omission of the 5 s lag period and more frequent sampling of arterial and cerebral venous blood during desaturation (Bernthman *et al.* 1978). This modification was used on animals of series B. Fig. 1 shows that, with this technique, it was possible to accurately assess the arterial and venous desaturation curves even when CBF exceeds $5 \text{ ml g}^{-1} \text{ min}^{-1}$. From curves obtained in normoxia, hypoxia and hypercapnia it could be estimated that the previous technique led to an overestimation of CBF by about 5% at low flows, and by about 15% at increased flows. These correction factors were used to recalculate previously published values (series A).

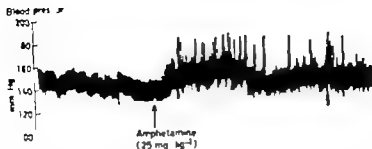
Arterial and cerebral venous samples for measurements of oxygen content (Co_2) were obtained just before desaturation, and about 1 min after start of desaturation. If arteriovenous differences in oxygen content (ΔVDO_2) did not differ by more than 10%, the mean value was multiplied with the calculated CBF to give CMR $_{O_2}$. If the difference was larger the experiment was discarded.

Regional CBF (rCBF) was measured with the technique of Lands *et al.* (1955 see also Riebel and Lands 1968), as applied to ^{14}C -ethanol (Eklöf *et al.* 1974). Infusion time was 30 s. Brain regions were dissected at $-10^\circ C$ before weighing.

Analytical techniques

Arterial PO_2 , PCO_2 and pH were measured with microelectrodes at $37^\circ C$, and corrections were applied for any difference in temperature between electrodes and animals. Arterial and cerebral venous oxygen and carbon dioxide were measured on 25 μl samples, using the polarographic method of Fabel and Lillibers (1964, see also Sjöström *et al.* 1974).

For studies of metabolite concentrations in blood and CBF samples were collected in liquid nitrogen. Following freezing of brain tissue *in situ*, pieces of frontoparietal cortex were dissected at $-22^\circ C$. Blood, CBF and tissue samples were extracted at $-22^\circ C$ with HCl-methanol, further extraction with perchloric acid was carried out at $0^\circ C$, and the neutralized extracts were subsequently analysed by thin layer chromatography, using the enzymatic, fluorometric techniques of Lowry and Passonneau (1972), in the same conditions as given previously (Folbergrova *et al.* 1972 and b, 1974 and c). The following metabolites were measured (in these): phosphocreatine (PCr), creatine (Cr), ATP, ADP, AMP, glycogen, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (FDP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PG), pyruvate, lactate, citrate, α -ketoglutarate, fumarate, malate, glutamate, aspartate, γ -aminobutyrate, GABA, alanine and β -alanine.



Typical blood pressure recording showing effects of intravenous injection of amphetamine (25 mg kg^{-1} over 60 min period).

tion was somewhat more pronounced, and there was a significant rise in lactate/pyruvate ratio. However at the end of 30 min, there was no statistically significant difference of lactate concentrations although the lactate/pyruvate ratio did differ ($p < 0.05$). CSF lactate and pyruvate concentrations were similar in control and amphetamine-injected animals.

Glucose distribution

Glucose concentrations in blood, CSF and tissue were measured 30 and 60 min after 5 mg kg^{-1} of amphetamine, as well as 30 min following 15 mg kg^{-1} (Table II). Intracellular concentrations were calculated on the assumption that blood and extracellular fluids occupied 3 and 15%, respectively of tissue volume. In general amphetamine had little effect on blood and CSF glucose concentrations. However there were consistent (but not significant) increases in intracellular to blood concentration ratios following 5 mg kg^{-1} and a significant increase after 15 mg kg^{-1} . Clearly the experiments failed to show that amphetamine reduces intracellular to blood glucose concentration ratios.

Tissue metabolites

Animals were frozen *in situ* for metabolite analyses following amphetamine in doses of 5 mg kg^{-1} *i.p.* (30 sec, 2 min and 5 min), 5 mg kg^{-1} *i.p.* (30 and 60 min) and 15 mg kg^{-1} *i.p.* (30 min). Results on cerebral energy state were consistent since no group of animals injected with amphetamine showed any variation from control in tissue concentrations of ATP, ADP or AMP. Cyclic AMP concentrations were measured 60 min after 15 mg kg^{-1} . The values ($1.81 \pm 0.04 \mu\text{mol g}^{-1}$) did not differ from those of the controls ($1.74 \pm 0.06 \mu\text{mol g}^{-1}$).

In animals that were frozen within the first 30 min following injection of amphetamine in doses of 2.5–5 mg kg^{-1} metabolic changes were relatively slight. Table III shows results obtained with 2.5 mg kg^{-1} *i.p.* (5 min), 5 mg kg^{-1} *i.p.* (30 min) and 15 mg kg^{-1} *i.p.* (30 min). Samples of animals from the last group were extracted against their own controls. In the table, the two control groups were pooled but statistical differences were calculated against the pooled control groups. Consistently there was a fall in glycogen concentration, and increases in G-6-P and α -KG concentrations. In animals given 2.5–5 mg kg^{-1} of amphetamine

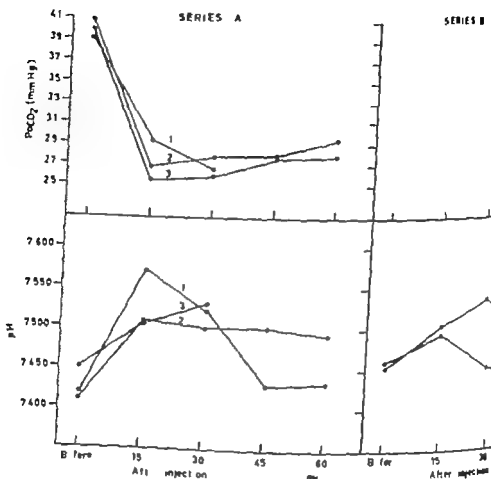
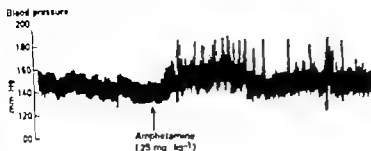


Fig. 2. Changes in arterial P_{CO_2} and pH in series A and B after intraperitoneal injection of amphetamine in spontaneously breathing rats.

crease was relatively short-lived and at 15, 30 or 60 min following *i.p.* or *i.v.* admin of amphetamine in doses of 2.5–15 mg kg⁻¹ blood pressure was close to control. Thus, the increases in CBF to be described below were unrelated to changes in pressure (or P_{CO_2}).

Temperature, mean arterial blood pressure, arterial P_{O_2} , P_{CO_2} and pH were measured in all groups (series A and B) used for measurements of CBF and CMR_{O_2} and in groups used for tissue metabolite analysis. All values were very similar to those of the control groups, except that both series showed a plasma acidosis with time, even though P_{aO_2} was controlled and P_{aCO_2} remained close to 40 mmHg.

Lactate and pyruvate concentrations were measured in blood and CSF 60 min after 5 mg kg⁻¹ of amphetamine, as well as 15 (blood) and 30 (blood and CSF) min after 15 mg kg⁻¹. Table I shows that following 5 mg kg⁻¹ of amphetamine, there was a slight increase in blood lactate concentration and lactate/pyruvate ratio. The mean CSF concentration of lactate was somewhat higher than in controls, but the difference was not statistically significant. Following 15 mg kg⁻¹ blood lactate concentrations in all animals rose somewhat between 15 and 30 min in all animals but there was no increase in CSF lactate. In amphetamine-intoxicated animals, the plasma lactate concentration



typical blood pressure recording showing effects of intravenous injection of amphetamine (2.5 mg kg^{-1} or 60 min period).

ion was somewhat more pronounced, and there was a significant rise in lactate/pyruvate ratio. However at the end of 30 min, there was no statistically significant difference in lactate concentrations although the lactate/pyruvate ratio did differ ($p < 0.05$). CSF and pyruvate concentrations were similar in control and amphetamine-injected

Glucose distribution

Glucose concentrations in blood, CSF and tissue were measured 30 and 60 min after 5 mg kg^{-1} of amphetamine, as well as 30 min following 15 mg kg^{-1} (Table II). Intracellular fractions were calculated on the assumption that blood and extracellular fluids occupy 3 and 15%, respectively of tissue volume. In general, amphetamine had little effect on blood and CSF glucose concentrations. However there were consistent (but not significant) differences in intracellular to blood concentration ratios following 5 mg kg^{-1} and significant after 15 mg kg^{-1} . Clearly the experiments failed to show that amphetamine reduces intracellular to blood glucose concentration ratios.

Time metabolites

Brain tissue was frozen *in situ* for metabolic analyses following amphetamine in doses of 2.5 mg kg^{-1} *l.p.* (30 sec, 2 min and 5 min), 5 mg kg^{-1} *i.p.* (30 and 60 min) and 15 mg kg^{-1} *i.p.* (60 min). Results on cerebral energy state were consistent since no group of animals injected with amphetamine showed any variation from control in tissue concentrations of GTP, ATP, ADP or AMP. Cyclic AMP concentrations were measured 60 min after 5 mg kg^{-1} . The values ($1.81 \pm 0.04 \mu\text{mol g}^{-1}$) did not differ from those of the controls ($1.4 \pm 0.06 \mu\text{mol g}^{-1}$).

In animals that were frozen within the first 30 min following injection of amphetamine in doses of 2.5–5 mg kg^{-1} metabolic changes were relatively slight. Table III shows results obtained with 2.5 mg kg^{-1} *l.p.* (5 min), 5 mg kg^{-1} *i.p.* (30 min) and 15 mg kg^{-1} *i.p.* (30 min). Some of animals from the last group were extracted against their own controls. In the table, two control groups were pooled but statistical differences were calculated against the pooled control groups. Consistently there was a fall in glycogen concentration, and increases in G-6-P and α -K-Glu concentrations. In animals given 2.5–5 mg kg^{-1} of amphetamine

TABLE I Lactate and pyruvate in arterial blood and CSF (in $\mu\text{mol g}^{-1}$) after i.p. administration of amphetamine compared to control groups. N O anaesthetics. n = number of animals. Mean \pm S.E.

Groups	n	Blood			Blood			CSF		
		Lac- tate	Pyru- vate 15 min	La/Py	Lac- tate	Pyru- vate 30 min	La/Py	Lac- tate	Pyru- vate 30 min	La/Py
Series A										
Control	6	2.17 ± 0.20	0.197 ± 0.005	11.0 ± 0.8	2.83 ± 0.28	0.211 ± 0.015	14.3 ± 2.7	2.49 ± 0.08	0.230 ± 0.003	10.8 ± 0.25
Amphetamine 15 mg kg ⁻¹	6	2.08 ± 0.27	0.187 ± 0.019	11.1 ± 0.9	3.46 ± 0.63	0.179 ± 0.017	19.3 ± 1.5	2.68 ± 0.21	0.236 ± 0.01	11.4 ± 0.25
Series B										
Control	6				1.42 ± 0.26	0.154 ± 0.021	9.2 ± 1.1	2.03 ± 0.10	0.170 ± 0.004	11.9 ± 0.44
Amphetamine 5 mg kg ⁻¹	6				3.14 ± 0.64	0.167 ± 0.011	17.4 ± 2.9	2.46 ± 0.17	0.236 ± 0.004	10.4 ± 0.25

the only other changes were reduction in DHAP (2.5 mg kg^{-1} i.p. 5 min) or 3-PG (5 mg kg^{-1} i.p., 30 min) concentrations. In spite of the fact that only some of the differences were statistically significant glycolytic changes appeared consistent since the mean values of G-6-P and F-6-P were increased and those for FDP, DHAP, GAP and 3-PG decreased. However, pyruvate and lactate concentrations remained constant unless the dose of amphetamine was increased to 15 mg kg^{-1} . In the latter group, both the lactate concentration and lactate/pyruvate ratio were slightly elevated.

In animals frozen 60 min after 5 mg kg^{-1} of amphetamine, metabolic changes were

TABLE II Glucose concentrations ($\mu\text{mol g}^{-1}$) in arterial blood, CSF, cortical tissue and calculated concentration in intracellular water (ICW), as well as ratios between ICW and blood, after amphetamine injection (i.p.) compared to control groups. n = number of animals.

Groups	n	Glucose				
		Blood	CSF	Tissue	ICW	ICW/B
Series A						
Control	6	12.6 ± 1.07	5.32 ± 0.23	4.32 ± 0.32	5.15 ± 0.45	0.41 ± 0.02
Amphetamine 15 mg kg ⁻¹ 30 min	6	10.00 ± 0.77	5.65 ± 0.33	4.09 ± 0.28	5.06 ± 0.37	0.49 ± 0.02
Series B						
Control	6	10.32 ± 0.46	5.95 ± 0.16	3.84 ± 0.19	4.31 ± 0.28	0.41 ± 0.02
Amphetamine 5 mg kg ⁻¹ 30 min	6	9.27 ± 0.64	5.03 ± 0.15	3.65 ± 0.25	4.28 ± 0.36	0.44 ± 0.02
Amphetamine 5 mg kg ⁻¹ 60 min	6	11.05 ± 0.84	5.43 ± 0.29	4.34 ± 0.40	5.24 ± 0.57	0.47 ± 0.02

The concentrations (mean \pm S.E.) of glycolytic, citric acid cycle metabolites and some associated amino acids in cerebral cortex after amphetamine administration. Values are given in $\mu\text{mol g}^{-1}$ number of animals.

L	Control 12	Amphetamine		
		2.5 mg kg ⁻¹ i. 5 min 3	5 mg kg ⁻¹ i.p., 30 min -6	15 mg kg ⁻¹ i.p., 30 min -6
	2.29 \pm 0.13	2.07 \pm 0.13	1.43 \pm 0.10***	1.40 \pm 0.08*
	4.08 \pm 0.24	4.15 \pm 0.21	3.65 \pm 0.25	4.09 \pm 0.28
	0.077 \pm 0.004	0.093 \pm 0.006	0.084 \pm 0.003	0.103 \pm 0.006
	0.011 \pm 0.001	0.012 \pm 0.001	0.012 \pm 0.001	
	0.121 \pm 0.002	0.115 \pm 0.003	0.114 \pm 0.002	
	0.024 \pm 0.001	0.021 \pm 0.001	0.022 \pm 0.001	
	0.023 \pm 0.001	0.022 \pm 0.004	0.029 \pm 0.002*	
	0.147 \pm 0.006	0.138 \pm 0.008	0.151 \pm 0.006	0.151 \pm 0.006
	1.83 \pm 0.06	1.83 \pm 0.16	1.73 \pm 0.07	2.34 \pm 0.16
	12.6 \pm 0.3	11.1 \pm 0.5	11.4 \pm 0.3	15.9 \pm 0.5*
	0.134 \pm 0.006	0.173 \pm 0.012	0.169 \pm 0.007*	0.192 \pm 0.012*
	0.069 \pm 0.003	0.070 \pm 0.002	0.072 \pm 0.002	
	0.410 \pm 0.016	0.411 \pm 0.025	0.419 \pm 0.013	0.445 \pm 0.032
	12.82 \pm 0.32	13.16 \pm 0.11	13.11 \pm 0.14	
	3.40 \pm 0.11	3.33 \pm 0.12	3.29 \pm 0.02	
	3.93 \pm 0.10	3.78 \pm 0.27	4.24 \pm 0.27	
	1.94 \pm 0.05	2.02 \pm 0.04	2.02 \pm 0.04	
	0.536 \pm 0.018	0.509 \pm 0.004	0.517 \pm 0.014	0.592 \pm 0.024
	0.272 \pm 0.023	0.252 \pm 0.018	0.229 \pm 0.018	0.262 \pm 0.029

joined (Table IV). Thus, there were significant increases in the concentrations of lactate and pyruvate, of all citric acid cycle intermediates except α -KG and of alanine, as well as a small decrease in GABA concentration.

CBF and CMR_{O₂}

The effects of amphetamine on CBF and CMR_{O₂} were not consistent between series A and B. In series A, our preliminary experiments indicated that amphetamine in doses of 2.5–5 mg kg⁻¹ gave rise to a 3-fold increase in CBF and to a 30–40% increase in CMR_{O₂} within the first 30 min, with further increases 60 min after 5 mg kg⁻¹ or 30 min after 15 mg kg⁻¹ (Carlsson *et al.* 1976; Benveniste *et al.* 1976). As Table V shows, there were differences in results between series A and B, especially in animals studied 30 min after 15 mg kg⁻¹ or 60 min after 5 mg kg⁻¹. These differences may reflect a difference in response to amphetamine between the two series (see Discussion). As judged from the (more precise) measurements obtained in series B, CMR_{O₂} increases by about 25% following amphetamine in doses of 2.5–5 mg kg⁻¹. Furthermore, the results provide some suggestion that the effects on CBF are dose-dependent in the range 0.25–25 mg kg⁻¹. With the largest dose, the CBF values obtained suggest that amphetamine may induce "maximal" vasodilatation in the brain (see Discussion). In order to validate the marked increase in CBF estimated by the ¹³³Xenon technique, and to obtain information of CBF distribution, regional CBF (rCBF) was evaluated from the autoradiographic uptake of ¹⁴C-ethanol (see Methods). Since this technique somewhat underestimates CBF, especially at high flows, rCBF was also measured in hypercapnia. As Table VI shows,

TABLE IV Glycolytic metabolites, citric acid cycle intermediates, associated amino acids, and glucose 60 min after amphetamine injection, compared to a control group. Values in $\mu\text{mol g}^{-1} \text{min}^{-1}$ \pm S.E. n = number of animals.

	Control n = 6	Amphetamine 5 mg kg^{-1} i.p., 60 min n = 6
glycogen	4.4 \pm 0.22	1.8 \pm 0.08
glucose	3.80 \pm 0.1	4.34 \pm 0.41
G-6-P	0.102 \pm 0.007	0.111 \pm 0.009
F-6-P	0.014 \pm 0.004	0.016 \pm 0.001
FDP	0.078 \pm 0.005	0.070 \pm 0.007
DIAP	0.039 \pm 0.003	0.039 \pm 0.002
3-PG	0.034 \pm 0.003	0.034 \pm 0.003
pyruvate	0.121 \pm 0.006	0.138 \pm 0.004
lactate	1.54 \pm 0.09	1.96 \pm 0.09
l-alpy	12.6 \pm 0.4	14.2 \pm 0.6
α -KG	0.153 \pm 0.009	0.176 \pm 0.013
fumarate	0.063 \pm 0.004	0.076 \pm 0.003
malate	0.343 \pm 0.021	0.407 \pm 0.010*
glutamate	12.88 \pm 0.13	13.31 \pm 0.15
aspartate	3.37 \pm 0.07	3.25 \pm 0.05
glutamine	5.67 \pm 0.18	5.43 \pm 0.23
GABA	2.58 \pm 0.05	2.38 \pm 0.05
ala. loc	0.457 \pm 0.020	0.518 \pm 0.015
ammonia	0.326 \pm 0.021	0.331 \pm 0.011

TABLE V Total arterial oxygen content (Tao_2), arteriovenous difference for oxygen (AVDO_2), cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMRO_2) in rats after administration of amphetamine in two series compared to control groups (means \pm S.E.), a number of animals.

Groups	n	Tao_2 $\text{ml } 100 \text{ ml}^{-1}$	AVDO_2 $\text{ml } 100 \text{ ml}^{-1}$	CBF $\text{ml } 100 \text{ g}^{-1} \text{ min}^{-1}$	CMRO_2 $\text{ml } 100 \text{ g}^{-1} \text{ min}^{-1}$
Series A					
Control	5	23.89 \pm 0.46	8.53 \pm 0.6	115 \pm 8	10.0 \pm 0.2
Amphetamine					
2.5 mg kg^{-1} i.v. 10 min	6	23.09 \pm 0.69	3.31 \pm 0.39*	453 \pm 85*	14.0 \pm 1.7***
5 mg kg^{-1} i.p. 15 min	4	23.02 \pm 0.87	3.86 \pm 0.27	315 \pm 40*	10.2 \pm 1.1
5 mg kg^{-1} i.p. 30 min	10	23.02 \pm 0.44	3.38 \pm 0.30*	388 \pm 37*	13.1 \pm 0.5***
5 mg kg^{-1} i.p., 60 min	6	23.3 \pm 1.31	3.99 \pm 0.46	482 \pm 59	19.2 \pm 0.8***
15 mg kg^{-1} i.p. 30 min	6	25.25 \pm 1.55	2.87 \pm 0.31	682 \pm 61	19.6 \pm 1.8***
Series B					
Control	9	22.09 \pm 0.27	8.42 \pm 0.51	112 \pm 13	8.9 \pm 0.4
Amphetamine					
0.25 mg kg^{-1} i. 10 min	4	22.59 \pm 0.27	4.06 \pm 0.62*	285 \pm 40*	10.9 \pm 0.5**
2.5 mg kg^{-1} i. 10 min	4	22.01 \pm 0.58	4.05 \pm 0.72	331 \pm 78*	11.7 \pm 1.5**
2.5 mg kg^{-1} i. 10 min	4	22.36 \pm 0.78	2.89 \pm 0.33	451 \pm 53	12.4 \pm 0.8**
2.5 mg kg^{-1} i.p., 10 min	4	20.99 \pm 1.17	3.05 \pm 0.55	345 \pm 46	9.5 \pm 0.8
2.5 mg kg^{-1} i.p., 30 min	4	22.65 \pm 0.86	4.23 \pm 0.92	274 \pm 16	11.0 \pm 1.3
5 mg kg^{-1} i.p., 30 min	5	22.71 \pm 1.24	4.61 \pm 0.90*	296 \pm 79*	11.1 \pm 1.6*
5 mg kg^{-1} i.p., 60 min					

data confirm those obtained with the ^{133}Xe technique in showing that amphetamine gives rise to a marked increase in CBF. However, in contrast to hypercapnia which increases CBF by similar degrees in all regions studied, amphetamine increased CBF much more in the frontal (and parietal) cortex than in other regions. It should be emphasized that, at a dose of 15 mg kg^{-1} of amphetamine, rCBF in frontal cortex was equal to that observed in hypercapnia.

Discussion

I discuss the present results under the headings (1) CBF and CMR_{O_2} , and (2) cerebral circulatory state.

CBF and CMR_{O_2}

The present results have confirmed our preliminary observation that amphetamine sulphate at doses of $5\text{--}15 \text{ mg kg}^{-1}$ gives rise to an increase of cortical CBF in paralyzed and artificially ventilated rats. However, the increase in flow was less pronounced than previously reported and, accordingly, there was only a moderate rise in CMR_{O_2} . In fact, some animals showed no increase at all in CMR_{O_2} . The results raise the question whether or not methodological factors are involved, i.e. if the previous CBF method grossly overestimated CBF at high flows encountered. As stated under Methods, the present modification of the ^{133}Xe technique involves omission of any lag in sampling of blood, following start of infusion, and more frequent sampling. This technique has now been applied to two high-flow situations, i.e. hypoxia and hypercapnia (Bernstein *et al.* 1978). The data indeed allow an approximate recalculation of CBF values obtained, using the previous "lag" procedure. The results show that the previously used technique can only have overestimated CBF by about 15%. Thus, differences in CBF and CMR_{O_2} between the present study and the previous one, can hardly be attributed to methodological factor; hence the differences point to a difference in response to amphetamine. In support of our assumption, the new strain showed some difference in response to amphetamine, including an attenuated rise in blood pressure and less pronounced hyperventilation.

Measurements of regional CBF using a completely different technique, corroborated the flow data obtained with the ^{133}Xe method, and these expts. gave the interesting information that CBF did not increase homogeneously; the most marked rise in CBF occurred in frontal, cortical regions. This is in contrast to hypercapnia (or hypoxia, see Salford & Sjöberg 1974) which is associated with a more uniform increase of rCBF. The results also showed that, following 15 mg kg^{-1} of amphetamine, frontal rCBF rose to values observed in animals maintained at an arterial P_{O_2} of about 75 mmHg. Thus, the increase in CBF induced by amphetamine is pronounced. It is of interest that amphetamine in doses of 5 mg kg^{-1} i.e. has recently been shown to induce blood-brain barrier dysfunction and that such extravasation is confined to frontoparietal regions (Carlsson and Johansson 1977). Previous studies in man (Abeu *et al.* 1949; Shenkman 1951) have failed to indicate that amphetamine increases CBF (or CMR_{O_2}) but the doses employed were much smaller than

TABLE VI Regional cerebral blood flow (ml 100 g⁻¹ min⁻¹) in different supra- and infratentorial areas. Arterial blood pressure (mmHg) and arterial CO₂ tension (mmHg) are also given. Treat-

Groups	MABP	PaCO ₂	Cortex			
			Frontal	Parietal	Tempo	Occip
Control	161 ±5	36.7 ±1.2	117 ±1	179 ±1	97 ±1	91 ±1
Hypercapnia	166 ±5	72.1 ±2.8	435 ±95	496** ±92	321** +41	496** ±92
Amphetamine 2.5 mg kg ⁻¹ I.v. 5 min	158 ±8	38.9 ±1.2	348 ±55	320* ±78	142* ±21	177* ±21
5 mg kg ⁻¹ I.p. 30 min	166 ±7	40.1 ±1.1	284 ±51	303 ±47	142 +25	284 ±51
15 mg kg ⁻¹ I.p., 30 min	170 ±9	40.1 ±1.2	467* ±91	274 ±31	142* ±28	284 ±51

those used presently. Since our preliminary observations were published, there have appeared 3 other reports, indicating that amphetamine may increase glucose consumption in the rat brain (Neuser and Hoffmeister 1977), or CBF and CMR_{glc} in the monkey brain (McCulloch and Harper 1977, McCulloch *et al.* 1977). CBF and CMR_{glc} were increased by 50 and 15 per cent, respectively after *i.v.* injection, while, in the latter study the results suggest that increases in CBF and CMR_{glc} are obtained only with small doses (0.75 nmol kg⁻¹ min⁻¹ by intracarotid infusion) while larger doses (250 nmol kg⁻¹ min⁻¹) have the opposite effect. In view of the differences in route administration, and in species, a direct comparison is difficult. However our results failed to indicate that such effects occur. Rather they show that effects on CBF and CMR_{glc} are, if anything, directly proportional to the dose.

In the expts. of Nahorski and Rogers (1973), energy flux was calculated from measured changes in the metabolites of the energy reserve, following decapitation of unanaesthetized mice. In view of the fact that there may have been changes in body temperature (see *cf.* Nahorski and Rogers 1975), the validity of the calculated energy flux values is not known. There are thus few data on cerebral metabolic rate in amphetamine-injected animals. The best interpretation of those available is that amphetamine induces a moderate increase in metabolic rate. Thus, CBF increases out of proportion to the rise in metabolic rate.

(2) Cerebral metabolic state

Published results on effects of amphetamine on cerebral metabolites are somewhat inconsistent. As remarked in the introduction, most workers have observed a decrease in glycogen concentration following amphetamine administration. The present results confirm these observations. In confirmation of some previous reports (Nahorski and Rogers 1975, King *et al.* 1975) our results also show that there were no changes in tissue concentrations of PCr or ATP. Our results offer the advantage that animals were studied after both short and long periods, following injection of amphetamine in *...* and that ATP

are compared to one control group and one hypercapnic group. Six animals in all groups. $\bar{x} \pm S.E.$

Values means	Cerebellum	Pons	Medulla spinalis
67	67	73	76
6	± 3	± 5	± 7
216 ^a	224 ^{ab}	254 ^{ab}	261
± 38	± 33	± 62	± 78
166	167 ^a	162	120 ^a
± 46	± 22	± 21	± 20
132 ^a	136 ^a	114 ^a	101
± 22	± 21	± 13	± 6
128	167 ^a	160	97
± 18	± 14	± 11	± 9

AMP concentrations were measured as well. Clearly amphetamine administration has an effect on cerebral energy state, as previously been reported that amphetamine administration is followed by increases in the concentrations of lactate and malate (Naborski and Rogers 1973, 1975). The increase in malate, but not in lactate, was confirmed by King *et al.* (1975) who also noted an increase in alanine concentration in mice, injected with amphetamine in a dose of 5 mg kg⁻¹. In view of the fact that amphetamine-injected animals may hyperventilate, and that hyperventilation by itself leads to increases in tissue concentrations of lactate, pyruvate, most acid cycle intermediates, and alanine (MacMillan and Siczjo 1973, Carlsson *et al.* 1975, Norberg 1976), such changes cannot without further proof be attributed to direct effects of amphetamine on brain metabolism.

In the present experiments, were conducted at constant P_{CO₂} (and body temperature), facilitating the assessment of amphetamine effects on metabolic pathways. Apart from the glycogenolysis, there were few effects of amphetamine in doses of 5 mg kg⁻¹ or less, when animals were sacrificed within 30 min following injection. The increases in G-6-P and a-KG concentrations cannot be interpreted with certainty. Thirty min following the administration of amphetamine in a dose of 15 mg kg⁻¹ there were moderate increases in lactate concentration and in the pyruvate ratio, and 60 min after a dose of 5 mg kg⁻¹ there was also an increase in the concentrations of pyruvate, measured citric acid cycle intermediates, and alanine. Presumably the increase in pyruvate concentration reflects a certain mismatch between rates of glycolysis and of pyruvate oxidation, while the increase in pyruvate by itself may explain the increase in citric acid cycle intermediates and alanine accumulation. This is because anaerobic conversion of pyruvate at the pyruvate carboxylase step depends on the pyruvate concentration, and because an increased pyruvate concentration usually leads to a shift in the malate dehydrogenase reaction towards alanine formation (see Norberg and Siczjo 1975).

Some of the effects observed after amphetamine administration, e.g. the decrease in glycogen concentration and the increases in the concentrations of pyruvate, lactate, citric acid cycle intermediates, and alanine, are similar to those occurring in seizures, e.g. those in-

duced by bicuculline. However in the latter condition the increased metabolic rate is a perturbation of the phosphorylation state of the tissue, and to a large degree is due to blood glucose concentration ratio (e.g. Chapman *et al.* 1977). In amphetamine-treated animals there was no change in cerebral energy state, and the tissue to blood glucose concentration ratio did not fall. These facts probably reflect the moderate increase in metabolic rate following amphetamine administration.

The authors are grateful to Gunilla Giddö for skilled technical assistance. This study was supported by grants from the Swedish Medical Research Council (Project No 14X 263), from U.S. 9513 Grant R01 NS07838-08 from NIH and Förenade Lf Insurance Company Stockholm.

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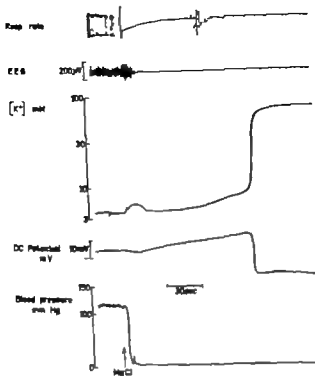
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1 Changes in the respiratory rate, EEG activity ($[K^+]_o$ in the brain cortex, DC-potential and in blood were in control rat following cardiac arrest induced by I. injection of 0.3 ml of saturated $MgCl_2$ solution. The ensuing cerebral ischemia evoked transient increase in $[K^+]_o$ concomitant with an augmented activity in the EEG. Following this event the rate of rise in $[K^+]_o$ became steadily faster and after 108 $[K^+]_o$ of 8.6 mEq there was rapid increase in $[K^+]_o$. At the same time the DC-potential showed a negative deflection. The EEG became flat after 18 min the respiratory activity stopped after 10 s, whereas other persons did not arrest.

preparation. They were bared overnight before the experiments. They were anesthetized with pentobarbital (50 mg/kg). A cannula was inserted into the trachea and thin polyethylene catheters were placed in the femoral artery and vein for recording of the blood pressure and for infusion, respectively. The body temperature was maintained at 37°C with a servo-controlled heating system. The animals were breathing spontaneously and the respiratory rate was recorded with a thermistor inserted in the tracheal tube. An ECG was recorded from needles in the two front legs. The head was immobilized in a stereotaxic frame and a trephine opening was made in the left parietal bone. The dura was carefully pierced with a sharp needle and a potassium-selective microelectrode was lowered into the cortex to a focal depth of approximately 0.4 mm by aid of a motor driven micromanipulator. Double-barreled potassium selective microelectrodes were made and used as described by Hansen (1977). The tip diameter was about 1 μ m and the impedance of the barrel containing the potassium exchanger (Corning 477317) was 10^8 – 10^9 Ohm. The response time (10%–90%) of the electrodes was 200–400 ms. The DC-potential was recorded between the reference barrel of the K⁺-electrode and the common ground placed in a neck muscle. The EEG was recorded differentially between 2 silver paste positioned on each side of the drilled hole and the common ground. The different parameters were recorded simultaneously on a multi-channel UV light recorder (Siemens Instrument).

Brain ischemia as produced by injection of 0.3 ml of saturated solution of $MgCl_2$ through the femoral vein. By this procedure the heart was arrested instantaneously without $MgCl_2$ reaching the capillary bed of the brain (Hansen 1977).

The extracellular potassium concentration in brain cortex following ischemia in hypo- and hyperglycemic rats

By

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Abstract

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The extracellular potassium concentration, $[K]_o$, was measured in the brain cortex of hypo- and hyperglycemic rats following brain ischemia. The increase in $[K]_o$ in control rats could be divided by 3 phases: an initial slow rate of rise where the $[K]_o$ rose in 1 min from 3 to 5 mM followed by a abrupt, steep increase to 60 mM within 10 s and finally a slow rise to 80 mM. In the hyperglycemic rats the same pattern appeared, but there were significant differences in the speed and duration of the initial phase which was approximately doubled in the hyperglycemic and halved in the ischemic group. The $[K]_o$ at which the steep increase was elicited was 8-10 mM in all groups. It is concluded that the duration of the initial phase is dependent upon available stores of glucose in the brain.

It has been shown that the extracellular potassium concentration, $[K]_o$, in the brain exhibits a large increase following anoxia (Vyskočil *et al* 1972, Morris 1974, Hansen 1977). The rise in the adult rat is characterized by an initial slow rate of rise followed by a steep increase, where the $[K]_o$ rises to 70 mM. Finally a slow rise to about 90 mM. The rate of rise was slower in the juvenile rat especially the duration of the initial phase was longer and inversely related to the age (Hansen 1977). The longer duration was due to lower metabolic demands of the juvenile brain and a greater tolerance of the brain to anoxia. In this way the brain might maintain a sufficient ATP level for a longer time thereby counteract the passive leak of potassium from the cells. However, since the structure and function of the nervous system rapidly changes during ontogenesis other factors might be important. A study was therefore undertaken to clarify the importance of the energy stores in the brain for the changes in $[K]_o$ during ischemia in the adult rat. A preliminary report has been given at the XXVIIIth International Congress of Physiological Sciences in Paris 1977.

Methods

The experiments were performed on male Sprague Dawley rats, weighing about 500 g. One group was made hyperglycemic with an i.p. injection of 3 ml 50% glucose, 30-45 min before ischemia. Another group was made hypoglycemic with an i.p. injection of 3 IU/kg of streptozotocin (Leo) 2.5 h before ischemia. Untreated rats served as controls. All rats were anesthetized with

1 $[K^+]_o$ in the rat cerebral cortex following ischemia. Effects of pretreatment with either glucose or insulin on the rise in $[K^+]_o$, EEG and respiratory activity following cardiac arrest (means \pm S.E., number of animals in parentheses).

	Hypoglycemia (5)	Control (7)	Hyperglycemia (6)
glucose concentration (mM)	$1.8 \pm 0.22^{**}$	6.6 ± 0.83	29.0 ± 3.6
$[K^+]_o$ (mM)	3.0 ± 0.49	3.1 ± 0.18	3.2 ± 0.3
at the start of the steep increase (mM)	8.1 ± 0.78	9.4 ± 0.48	9.5 ± 0.4
rate of rise in $[K^+]_o$ (mM-sec $^{-1}$)	76 ± 11	74 ± 3.4	$20 \pm 2.8^*$
$[K^+]_o$ (mM)	75 ± 1.6	79 ± 2.0	74 ± 1.5
until start of the steep increase (sec)	$39 \pm 4.3^{***}$	105 ± 6.9	208 ± 11.1
are until last EEG (sec)	$14 \pm 1.0^{**}$	19 ± 0.9	$22 \pm 0.6^{**}$
are until final gasp (sec)	$52 \pm 1.3^*$	82 ± 2.7	10 ± 7.1

are different from control groups: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

terminal value of $[K^+]_o$. The most pronounced difference was found in the duration of the initial, slow increase in $[K^+]_o$. In the hyperglycemic group the initial phase was as long as in the controls while in the hypoglycemic group it was less than one half. The highest rate of rise during the steep increase, which in all groups was attained at a $[K^+]_o$ of 30-40 mM, was similar in the normo- and the hypoglycemic groups, but significantly lower in the hyperglycemic group. The duration of the EEG during ischemia and time to the last gasp were also influenced by the pretreatment.

Discussion

This study has shown that altering the plasma glucose level alters the rate of rise of the initial, slow increase and the steep increase in $[K^+]_o$ without changing the $[K^+]_o$ at which the steep increase begins. In the following the two phases will be discussed separately.

slow increase

The variation in the plasma level of glucose was induced as described by Ljunggren *et al.* (1974). They showed in similarly pretreated rats that the brain glucose level increased 3-fold with the given glucose and decreased to about 1/10 of the normal value in those receiving insulin. Since glucose is an important energy substrate during anaerobic conditions, release of ATP should be different in the three groups. Accordingly Ljunggren *et al.* (1974) showed that the rate of lactate accumulation in the brain cortex following ischemia, reflecting the anaerobic production of ATP, was larger in the hyperglycemic rats and lower in the hypoglycemic rats, than in controls. In another study Hansen and Nordström (in preparation) compared the $[K^+]_o$ in the brain cortex and the level of cortical energy stores (phosphocreatine, ATP, ADP, glucose and glycogen) during ischemia in normal juvenile rats and juvenile rats with a high level of glucose in the brain. They found that the rate of rise in $[K^+]_o$ was lower and the cortical energy consumption, measured as the rate of decline in energy stores, concomitantly was higher in the group with high brain glucose level, than in controls, suggesting that the lower rate of rise in $[K^+]_o$ was due to a higher activity of ion

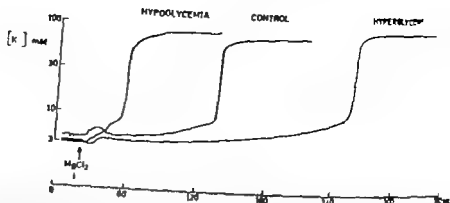


Fig. 2. Examples of recordings of $[K]_b$ in the brain cortex of three rats following cardiac arrest by i.v. injection of a saturated $MgCl_2$ solution. In order to change the glucose stores in the brain we pretreated with either glucose or insulin. The rate of rise in $[K]_b$ following cerebral ischemia was dependent upon the pretreatment. Note that the $[K]_b$ at the beginning of the steep increase is the same in all three animals.

After the electrode was inserted into the brain cortex at least 3 min elapsed at which the potassium concentration was constant. A few minutes before the induction of ischemia 0.5 ml of blood was sampled from the femoral artery. The glucose concentration in the serum was determined by a glucose oxidase test. The results were statistically evaluated using Student's *t*-test.

Results

Fig. 1 shows an expt. from a control rat where brain ischemia was induced without previous changes in the plasma glucose level. The resting level of $[K]_b$ was 3.6 mM. Following ischemia the $[K]_b$ level initially exhibited a transient increase, presumably due to release of potassium from the nerve cells as evidenced by the augmented amplitude in the EEG. After about 1 min there was a progressive rate of rise in $[K]_b$ and after 2 min, at a $[K]_b$ of 9 mM a rapid rise took place. During this event the $[K]_b$ increased to 60 mM within 1 min. From then on the rate of rise became slower and a terminal value of 85 mM was reached. The DC-potential showed a positive-going shift during the initial rise in $[K]_b$ (see below). During the transient increase in $[K]_b$ the DC-potential showed a negative shift, so-called anoxic depolarization (Leão 1947). The EEG exhibited an increased amplitude immediately following the injection of $MgCl_2$ and became flat after 18 s. The respiration stopped after 10 s. After a pause it started again with a series of gasps.

Fig. 2 shows the change in $[K]_b$ following ischemia in individual rats belonging to the three groups. It can be seen that the initial rise is clearly dependent upon the pretreatment. Thus, both the duration and the rate of rise was influenced by the pretreatment. Despite a significant difference in the duration of the initial phase the steep increase was elicited in all groups when the $[K]_b$ had reached a value of 8–10 mM.

Table I summarizes the different parameters measured in the three groups prior to and during ischemia. The treatment with either glucose or insulin induced large changes in the plasma glucose level. On the other hand, it did neither affect the resting level of $[K]_b$ nor the $[K]_b$ at which the steep increase started during ischemia. Nor were there any differences

There are several suggestions as to a possible mechanism behind the dramatic increase in $[K^+]_o$, but real insight into the chain of events is lacking.

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pumping. Accordingly the inverse relation between the rate of rise in $[K^+]_o$ and the plasma glucose level observed in this study is probably caused by differences in ion pump activity.

Steep Increase

Since the length of the initial phase was related to the plasma glucose level, and hence to the glucose level in the brain, it is possible that the steep increase was elicited by a complete stop of ion pumping due to substrate failure. Data on this matter is, however, lacking. It is tempting to try to assess the changes in $[K^+]_o$ caused by termination of ion pump activity.

Suppose that 90% of the ATP hydrolysis in the brain cortex ($30 \mu\text{mol g}^{-1} \text{min}^{-1}$; B. F. et al 1973) is used for maintaining ion gradients. The Na⁺-K⁺ pump uses 1 ATP molecule for transporting 3 sodium ions out of the cell and 2 potassium ions into the cell. The pump then counteracts a leak of potassium from the cells which in an extracellular space of 10^{-4} would lead to an increase of $[K^+]_o$ with a rate of 6 mM s^{-1} ($30 \cdot 0.9 \cdot 2 \cdot 10^{-4}$). The calculated figure is clearly lower than the experimentally determined rate of rise (cf. Table 1). It is obviously only valid for the assumed size of the extracellular space and for the membrane potential and the membrane permeability to potassium existing in the unanesthetized cortex. Changes in these parameters would affect the rise in $[K^+]_o$. The extracellular space in the brain shrinks to 1/2-1/3 during anoxic depolarization (i.e. the steep increase) (de Harreveld 1972), but this change is too small to account for the observed rate of rise in $[K^+]_o$. Changes in ionic permeability of the cell membrane is another possibility. An overall increase in potassium permeability cannot explain the steep rise since potassium is distributed across the cell membranes near electrochemical equilibrium, and net-potassium flux is therefore small until significant depolarization has taken place. A depolarization of the cells would require sodium influx. In accordance with this, during a spreading depression, where a similar steep increase in $[K^+]_o$ is observed (Vyskočil et al 1972) there is a considerable decrease in the extracellular sodium concentration (Kraig and Nicholson 1976). Thus an increase in the sodium permeability could provide a basis for the steep increase. This increase in sodium permeability could be mediated by the increase in $[K^+]_o$ depolarizing the nerve cells (Hodgkin and Huxley 1952). The observation that the $[K^+]_o$ was the same in all groups at the beginning of the steep increase supports this idea. The "threshold" value of $2-40 \text{ mM}$ is in accordance with the findings of Vyskočil et al (1972) who produced brain smooth by respiratory arrest and with Hansen (1977) who used nitrogen inhalation. The fact that when the $[K^+]_o$ in the brain cortex exceeds $10-12 \text{ mM}$ during electrical stimulation a spreading depression is elicited (Lothmann et al 1975) also corroborates this view.

The reason why the maximal rate of rise was considerably lower in the hyperglycaemic rats than in the other groups is so far unknown. It might be speculated that the population of cells which delivers the potassium during the steep increase is inhomogeneous. When the initial phase is prolonged the release of potassium occurs at different times leading to a lower rate of rise.

This discussion has focused upon potassium as the mediator of the steep increase, but the possibility remains, however, that the metabolic breakdown damages the membrane integrity thus leading to unselective permeability changes striking glial cells as well as neurons.

was exclusively carried by Ca ions. The spike frequency increased by application of tropic releasing hormone (TRH), which increases prolactin secretion from these cells (Dames *et al.* (1977) have also reported that cultured cells from anterior pituitary gland, including the GH line, human acidophilic and chromophobic adenoma, generate potentials. However the action potentials of the GH cells were in this case shown to be both Ca- and Na-dependent components.

In these studies on neoplastic tumour cells from the anterior pituitary gland two questions emerge: (1) Have normal anterior pituitary cells the ability to generate action potentials? (2) If so, which ions act as inward current carriers?

We have examined the membrane electrical properties of anterior pituitary cells of the rat. The present results show that most anterior pituitary cells are electrically excitable, and that the action potentials are partly dependent on Ca ions. A brief report on the present results has already appeared (Ozawa and Sand 1977).

Methods

Anterior lobe of the pituitary gland was removed from female rats (Wistar strain) weighing 200–250 g. Slices of 300–500 μ m thickness were prepared from each hemisphere of the anterior lobe. The slices were essentially similar to that commonly used for making hippocampal slices. From this latter preparation it is possible to obtain in some intracellular recordings from the pyramidal cells showing action potentials and synaptic potentials which do not differ from those observed *in vivo* (Yasumoto 1972). Prior to experiments the slices were incubated at 37°C for 2 h in a buffer gassed with either mixture of 95% O₂ and 5% CO₂ or pure oxygen. A selected slice was then transferred to the 0.4 ml superfusion chamber where the slice was clamped on a stainless steel mesh covering the outlet hole at the bottom. A slow rate of the oxygen-saturated buffer through the chamber as 1 ml/min, and the position of the slice facilitated solution exchange when the slice. The temperature of the perfusing solution was raised at 37 ± 1°C.

The composition of the normal buffer was NaCl 124 mM, KCl 5 mM, NaH₂PO₄ 1.24 mM, NaHCO₃ 24 mM, MgCl₂ 1.3 mM, CaCl₂ 2.4 mM and glucose 10 mM. This solution was gassed with 95% O₂ and 5% CO₂. Most experiments were, however, performed in standard buffer of the following composition: NaCl 120 mM, KCl 5 mM, MgCl₂ 1.3 mM, CaCl₂ 2.4 mM, glucose 10 mM and buffered by Tris-HCl 5 mM, pH 7.4. This solution was gassed with pure oxygen. No difference was observed between the results obtained in these two solutions. Na-free solutions were made by replacement of NaCl of the standard solution by choline-Cl. Solution with high Ca²⁺ concentration was prepared by substitution of either Cl⁻ or choline-Cl for CaCl₂ on an ionic basis. Ba and Sr solutions were made by replacement of Ca₂ with BaCl₂ and SrCl₂, respectively.

Micro-electrodes for recording cellular transmembrane potentials were filled from behind by silbury action with 4 M K-acetate solution adjusted to pH 7.2 with acetic acid. The electrode resistance varied between 70 and 120 M Ω . The electrodes are connected to a high input impedance preamplifier with the facility for injecting current through the recording electrode using bridge circuit (Bioelectronic Laboratory AM 1). The input resistance of the preamplifier was more than 10¹¹ M Ω and input offset current was less than 10⁻¹⁰ A. The time derivative of the potential change was recorded on electronic differentiation circuit.

Results

Electrophysiological responses in standard solution

Inward current pulses of 200–300 ms duration and less than 1 $\times 10^{-10}$ A intensity were continuously injected during advancement of the microelectrode through the anterior pituitary slice. Successful penetration of a cell was noticed by sudden appearance of the

Electrical activity of rat anterior pituitary cells *in vitro*

By

SEIJI OZAWA and OLAV SAND

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Abstract

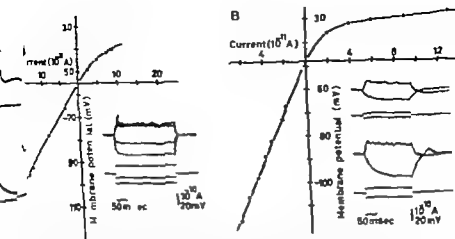
OZAWA, S and O SAND *Electrical activity of rat anterior pituitary cells in vitro* Acta physiol scand. 1978 102 330-341

Regenerative responses were seen in most cells either after cessation of an inward current pulse or during an outward current pulse. Two cell groups were distinguished electrophysiologically. Type I cells showed action potentials with maximum rate of rise of 21.2 ± 9.0 V/sec (mean \pm S.D. $n=19$). Type II cells generated small graded depolarizing responses with maximum rate of rise less than 3 V/s. The action potentials of type I and II cells were 31.8 ± 14.9 mV ($n=19$) and 41.7 ± 9.8 mV ($n=37$), respectively. The steady-state current/voltage relationship was linear for both cell types when the membrane potential was more negative than -60 mV. An outward rectification appeared when the membrane potential was more positive than -40 mV. The input resistance was smaller in type I cells (274 ± 212 M Ω , $n=19$) than in type II cells (111 ± 456 M Ω , $n=16$). Even in Na-free solution regenerative responses were observed in most cells. When the Ca^{2+} concentration was increased tenfold to 4 mM, the maximum rate of rise of the off-response increased from 1.9 ± 0.8 V/s ($n=11$) to 5.7 ± 2.12 V/s ($n=5$). All-or-none action potentials could be evoked in this Ca^{2+} -rich solution. Action potentials of similar maximum rate of rise could be evoked after replacing 4 mM Ca^{2+} with isomolar Sr^{2+} . Prolonged action potentials were seen after substitution of Ca^{2+} for Ba. It is concluded that action potentials in most anterior pituitary cells have a Ca component, which in type I cells is additional to a Na component.

Key words: Anterior pituitary action potential, Ca spike, hormone secretion.

It has recently been demonstrated that endocrine cells from the pancreatic islets (Aebi and the adrenal medulla (chromaffin cells) are capable of generating action potentials (Dean and Matthews 1968, 1970 a, Blake *et al* 1976). These action potentials are due at least partly to an increase of the membrane permeability to Ca^{2+} ions (Dean and Matthews 1970 b, Brandt *et al* 1976). Presence of Ca^{2+} ions in the extracellular solution is indispensable for hormone secretion, and it is generally accepted that entry of external Ca^{2+} ions into the cells triggers exocytosis of the secretory granules. It is therefore reasonable to suggest that the Ca^{2+} spike-generating mechanism of these cell membranes has important functional significance in the stimulus-secretion coupling.

Regarding the anterior pituitary cells, Kidokoro (1975) observed spontaneously occurring spikes in a clonal cell line (GH $_3$) isolated from a rat anterior pituitary tumour which continuously secretes growth hormone and prolactin. He concluded that the inward action



Steady-state current/voltage relations for type I (A) and type II (B) cells in standard saline. The open potential changes measured at the end of the current pulses were plotted. Inward current is up on the abscissa. The resting potential was -55 mV and -48 mV in A and B, respectively. The resistance estimated from the linear portion of the I/V relation was 275 M Ω in A and 1080 M Ω in B. Sample records showing time course of the potential change. The potential difference across recording electrode was not completely cancelled in record A, and corrected values were therefore used on the graph.

ward current pulses in type II cells, but small graded responses with the appearance of potential oscillations were instead superimposed on the electrotonic potential. The resting potentials of type II cells were more negative (41.7 ± 9.8 mV, $n=31$) and generally more negative than for type I cells. The penetrations frequently lasted for several minutes.

Input resistance and capacitance

A steady state relation between the current intensity and the resulting voltage deviation at the end of injected current pulses of 200–300 msec duration was obtained for both type I and II cells in standard saline (Fig. 2A & B). The relation was linear for both groups as the membrane potential was more negative than -60 mV and the slope resistance tended to decrease when the membrane potential was made more positive than -40 mV. Anterior pituitary cells thus have the property of outward rectification seen in most excitable cells. This outward rectification was more pronounced in type II cells. The input resistance was estimated from the linear portion of the current/voltage relation. Although the values for type I cells scattered widely from 92 M Ω to 960 M Ω (274 ± 212 M Ω , $n=19$), this is in the same range as the values reported by Kidokoro (1975) and Biales *et al.* (1977) for other clonal cell lines. There was a clear correlation between the resting potential and the input resistance in type I cells. The input resistance decreased with less negative resting potentials, probably due to increase in leak conductance following cell damage caused by the penetration. Hence, the mean value of the input resistance of type I cells described above is possibly an underestimation of the true value. The resting potentials of type II cells were less scattered and there was no correlation between the resting potential and the

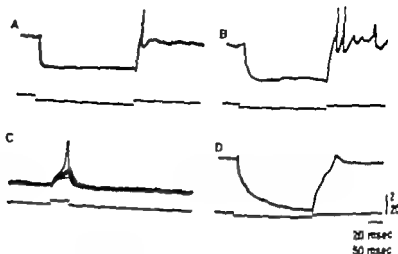
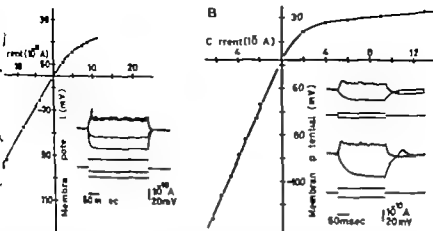


Fig. 1 Intracellular records of regenerative responses in standard saline. A and B: responses (type I) elicited at the termination of an anodal current pulse. C: action potential during short outward current pulse. D: slow regenerative response (type II). The time scale is applicable only to record C.

negative DC membrane potential and the hyperpolarising electrotonic potential. In about 80% of the penetrated cells where these fulfilled regenerative responses were evoked either at the termination of the pulse or during an outward current pulse (Fig. 1). However the regenerative responses in the type II cells described below were rather fragile, and frequently faded when the electrode was still inside the cell. It is therefore doubtful if the remaining penetrated cells were really non regenerative. The present paper deals only with a majority of cells which displayed regenerative responses.

The cells could be divided into two classes according to the characteristics of their regenerative responses. In one group of cells a regenerative spike with an amplitude of 45 mV (mean \pm SD -29.1 ± 10.3 mV, $n=19$) was evoked at the termination of a current pulse (Fig. 1A). The maximum rate of rise of the response ranged from 40 V/s (21.2 ± 9.0 V/s, $n=19$). Repetitive firing was seen in some cases (Fig. 1B) and was followed by afterhyperpolarization. The resting potential in these cells was usually in the range of -20 to -50 mV (31.8 ± 14.9 mV, $n=19$). The hyperpolarization of the resting potential was possibly due to cell injury caused by the electrode. In agreement with this interpretation, the recordings from these cells were rare and usually lasted less than 1 min. However three cells had resting potentials of -55 and -65 mV and in these cells all-or-none action potentials could be evoked by outward current pulses in a similar way as usually seen in other excitable cells. The cells capable of generating fast rising action potentials with maximum rates of rise of more than 11 V/s will tentatively be referred to as type I cells in the present paper.

Much smaller and more slowly rising graded regenerative responses were evoked at the termination of the current pulse in the remaining responsive cells (Fig. 1D). These will tentatively be called type II cells. The amplitude of these responses ranged between 5 and 15 mV and the maximum rate of rise was less than 3 V/s. Action potentials were not



Steady-state current/voltage relations for type I (A) and type II (B) cells in standard saline. The membrane potential changes measured at the end of the current pulses were plotted. Inward current is plotted as the abscissa. The resting potential was -35 mV and -48 mV in A and B, respectively. The resistance estimated from the linear portion of the I/V relation was 275 M Ω in A and 1080 M Ω in B. Sample records showing trace course of the potential change. The potential difference across the electrode is not completely cancelled in record A, and corrected values were therefore used in the graph.

and current pulses in type II cells, but small graded responses with the appearance of small oscillations were instead superimposed on the electrotonic potential. The resting potentials of type II cells were more negative (41.7 ± 9.8 mV, $n=31$) and generally more stable than for type I cells. The penetrations frequently lasted for several minutes.

Input resistance and capacitance

The steady state relation between the current intensity and the resulting voltage deviation at the end of injected current pulses of 200–300 msec duration was obtained for both type I and type II cells in standard saline (Fig. 2A & B). The relation was linear for both groups when the membrane potential was more negative than -60 mV and the slope resistance decreased when the membrane potential was made more positive than -40 mV. Anterior pituitary cells thus have the property of outward rectification seen in most excitable cells. This outward rectification was more pronounced in type II cells. The input resistance was estimated from the linear portion of the current/voltage relation. Although the values for type I cells scattered widely from 92 M Ω to 960 M Ω (274 ± 212 M Ω , $n=19$), this is the same range as the values reported by Kikukoro (1975) and Biales *et al.* (1977) for clonal cell lines. There was a clear correlation between the resting potential and the input resistance in type I cells. The input resistance decreased with less negative resting potentials, probably due to increase in leak conductance following cell damage caused by penetration. Hence, the mean value of the input resistance of type I cells described above is possibly an underestimation of the true value. The resting potentials of type II cells were less scattered and there was no correlation between the resting potential and the

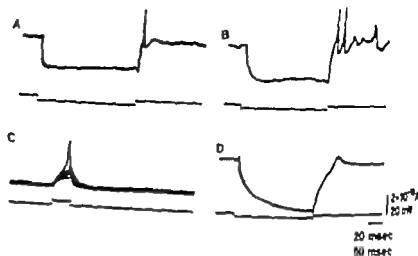


Fig. 1 Intracellular records of regenerative responses in standard saline. A and B, fast regenerative responses (type I) elicited at the termination of an anodal current pulse. C, action potential evoked by a short outward current pulse. D, slow regenerative response (type II). The time scale of 50 msec is applicable only to record C.

negative DC membrane potential and the hyperpolarising electrotonic potential caused by the inward current pulse. In about 80% of the penetrated cells where these criteria were fulfilled regenerative responses were evoked either at the termination of the inward current pulse or during an outward current pulse (Fig. 1). However the regenerative responses in the type II cells described below were rather fragile, and frequently faded away when the electrode was still inside the cell. It is therefore doubtful if the remaining 20% of penetrated cells were really non regenerative. The present paper deals only with the majority of cells which displayed regenerative responses.

The cells could be divided into two classes according to the characteristics of their regenerative responses. In one group of cells a regenerative spike with an amplitude of 45 mV (mean \pm S.D. = 29.1 ± 10.3 mV, $n=19$) was evoked at the termination of an inward current pulse (Fig. 1A). The maximum rate of rise of the response ranged from 11 to 40 V/s (21.7 ± 9.0 V/s, $n=19$). Repetitive firing was seen in some cases (Fig. 1B). These responses were followed by afterhyperpolarisation. The resting potential of these cells were usually in the range of -20 to -50 mV (31.8 ± 14.9 mV, $n=19$). The low value of the resting potential was possibly due to cell injury caused by the electrode penetration. In agreement with this interpretation, the recordings from these cells were rather unstable and usually lasted less than 1 min. However three cells had resting potentials between -55 and -65 mV and in these cells all-or-none action potentials could be generated by outward current pulses in a similar way as usually seen in other excitable cells (Fig. 1C). The cells capable of generating fast rising action potentials with maximum rate of rise of more than 11 V/s will tentatively be referred to as type I cells in the present paper.

Much smaller and more slowly rising graded regenerative responses were evoked at the termination of the current pulse in the remaining responsive cells (Fig. 1D), which will tentatively be called type II cells. The amplitude of these responses ranged between 3 and 10 mV and the maximum rate of rise was less than 3 V/s. Action potentials were not evoked.

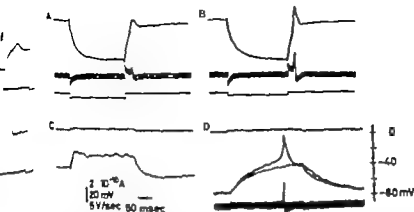


Figure 4. Regenerative responses in Na-free solution. A, response to anodal current break in 2.4 mM Ca^{2+} . B, corresponding response recorded from the same slice 45 min after introduction of 24 mM Ca^{2+} . C, membrane potential change in response to cathodal current pulse in 24 mM Ca^{2+} solution after penetration. D, an all-or-none action potential was elicited by cathodal current pulse of 33 mV into the membrane was hyperpolarized by 33 mV. The middle trace in A and B and the trace in D give the first order derivative of the potential change.

excitation was in these exper. performed to wash out Na ions completely. Several cellular recordings from a selected slice were then obtained in this solution, where the slice was superfused with Na-free solution containing 24 mM Ca^{2+} (10 Ca^{2+} too) for at least 30 min before the recordings were repeated. Both the amplitude and maximum rate of rise of the regenerative response increased remarkably in the Na-free solution of high Ca^{2+} concentration, as illustrated in Fig. 4 A and B. The largest value of the maximum rate of rise of the graded regenerative response which occurred at the initiation of the inward current pulse was $1.9 \pm 0.8 \text{ V/s}$ ($n=11$) in normal Ca^{2+} concentration and $57 \pm 2.1 \text{ V/s}$ ($n=5$) in 10 Ca^{2+} solution. Furthermore, all-or-none action potentials could be evoked by depolarizing pulses in the Na-free solution of high Ca^{2+} concentration when the membrane potential was hyperpolarized to a level more negative than -75 mV by background current. The cell illustrated in Fig. 4 had a resting potential of -44 mV shortly after the penetration and showed small graded depolarizations in response to an outward current pulse (Fig. 4 C). When the membrane potential was shifted to -77 mV by continuous inward current, an all-or-none action potential was generated by a outward current pulse (Fig. 4 D). The action potential was unaffected by application of 1 μM tetrodotoxin.

Action potentials in Sr and Ba solution

Among the most excitable tissues exhibiting a Ca-dependent regenerative response, Sr^{2+} and Ba^{2+} are substitutes for Ca^{2+} as current carrier (Fatt and Ginsborg 1958). We therefore conducted experiments in which the 24 mM Ca^{2+} in Na-free solution was replaced with 4 mM Sr^{2+} or Ba^{2+} . Regenerative responses similar to those observed in 4 mM Ca^{2+} solution are recorded at the termination of the anodal current pulse in the Na-free

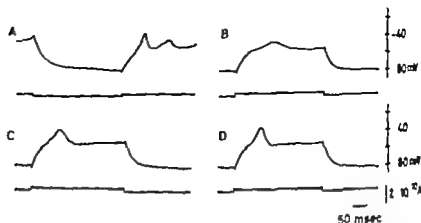


Fig. 3 Depolarising responses in Na-free solution containing 2.4 mM Ca^{2+} . A, representative action potential recorded shortly after penetration. B, C and D records obtained while the membrane potential was held at -78 mV by DC hyperpolarising current. The magnitude of the graded responses increased with increasing intensity of the applied cathodal current pulses.

input resistance. These results probably reflect that type II cells were less damaged by penetration. The input resistance of type II cells averaged $1112 \pm 456 \text{ M}\Omega$ ($n=16$), which is four times larger than the underestimated value for type I cells.

The membrane time constants for the two cell types were estimated from the exponential time course of the potential change produced by a small inward current pulse (see sample records in Fig. 2). The values were $4.6 \pm 3.0 \text{ ms}$ ($n=19$) and $29.1 \pm 12.6 \text{ ms}$ ($n=10$) for type I and II cells, respectively. The corresponding membrane capacities obtained from the time course and the membrane resistance were $17 \pm 10 \text{ pF}$ ($n=19$) and $2.6 \pm 1.6 \text{ pF}$ ($n=16$).

Regenerative responses in Na-free solution

After superfusion of the thin slice with Na free solution containing 2.4 mM Ca^{2+} for several minutes, fast rising regenerative responses similar to those of type I cells in normal buffer were never observed. However the cells were still excitable in Na free solution. Successful penetrations of numerous cells with resting potentials of about -40 mV were performed, and a small but significant regenerative response almost always occurred at the termination of the inward current pulse (Fig. 3 A). In the cell illustrated in Fig. 3 the membrane potential was shifted from -44 mV to -78 mV by a continuously injected DC hyperpolarising current. Outward current pulses of increasing intensity were then applied, and Fig. 3 B-D show how these pulses produced significant depolarising responses of increasing magnitude superimposed on the passive electrotonic potential. Action potentials of all-or-none type could not be evoked in Na-free solution containing 2.4 mM Ca^{2+} but the presence of a graded response suggests that a potential dependent increase of the membrane permeability to Ca^{2+} may occur in anterior pituitary cells.

In order to test this possibility the effects of increased Ca^{2+} concentration was studied. Pre incubation of the sliced anterior pituitary for 3 h in Na-free solution containing 2.4 mM Ca^{2+} was studied.

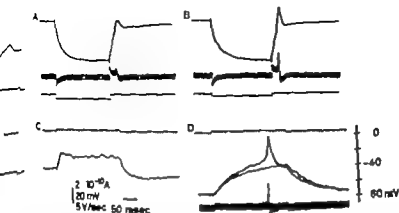


Fig. 4. Regenerative responses in Na-free solution. A, response to anodal current break in 2.4 mM Ca^{2+} . B, corresponding response recorded from the same slice 45 min after introduction of 4 mM Ca^{2+} . C, membrane potential change in response to cathodal current pulse in 24 mM Ca^{2+} solution after penetration. D, an all-or-none action potential was elicited by a cathodal current pulse of steady state. The membrane was hyperpolarized by 33 mV. The middle trace in A and B and the trace in D give the first order derivative of the potential change.

situation was in these expts. performed to wash out Na ions completely. Several cellular recordings from a selected slice were then obtained in this solution, where the slice was superfused with Na-free solution containing 24 mM Ca^{2+} (10 Ca^{2+} ions) for at least 30 min before the recordings were repeated. Both the amplitude and maximum rate of rise of the regenerative response increased remarkably in the Na-free solution of high Ca^{2+} concentration, as illustrated in Fig. 4 A and B. The largest value of the maximum rate of rise of the graded regenerative response which occurred at the termination of the anodal current pulse was 1.9 ± 0.8 V/s ($n = 11$) in normal Ca^{2+} concentration and 5.7 ± 2.1 V/s ($n = 5$) in 10 Ca^{2+} solution. Furthermore, all-or-none action potentials could be evoked by depolarizing pulses in the Na-free solution of high Ca^{2+} concentration when the membrane potential was hyperpolarized to a level more negative than -75 mV by background current. The cell illustrated in Fig. 4 had a resting potential of -44 mV shortly after the penetration and showed small graded depolarizations in response to an outward current pulse (Fig. 4 C). When the membrane potential was shifted to -71 mV by continuous inward current, an all-or-none action potential was generated by a outward current pulse (Fig. 4 D). The action potential was unaffected by application of 1 μM tetrodotoxin.

Regenerative potentials in Sr and Ba solution

Most excitable tissues exhibiting a Ca-dependent regenerative response, Sr^{2+} and Ba^{2+} as substitutes for Ca^{2+} as current carrier (Fatt and Ginsborg 1958). We therefore conducted experiments in which the 24 mM Ca^{2+} in Na-free solution was replaced with 20 mM Sr^{2+} or Ba^{2+} . Regenerative responses similar to those observed in 24 mM Ca^{2+} solution were recorded at the termination of the anodal current pulse in the Na-free

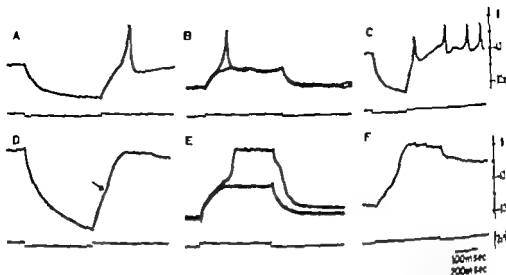
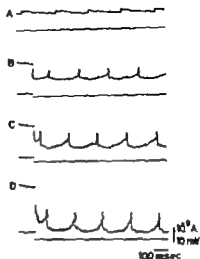


Fig. 5 A, B and C, action potentials in Na free, 24 mM Sr^{2+} solution. A, action potentials evoked at termination of an anodal current pulse. B, all-or-none action potentials elicited by a cathodal current pulse after the membrane potential was shifted to -75 mV by DC hyperpolarizing current. C, example of repetitive firing of a different cell after cessation of an anodal current pulse. The time scale of 200 ms is applicable only to this record. D, E and F regenerative responses in Na-free, 4 mM Sr^{2+} solution. D, anodal break response. Arrow indicates the inflexion reflecting the critical membrane potential for a regenerative response. Note that the resting potential was positive in this cell. E, prolonged action potential elicited by an outward current pulse after the membrane potential was shifted to -75 mV. F, prolonged regenerative response lasting about 1 s.

solution containing 24 mM Sr^{2+} (Fig. 5 A). The largest value of the maximum rate of rise of the regenerative response was 5.9 ± 2.5 V/s ($n=9$). Repetitive firing occasionally occurred after the anodal pulse, as shown in Fig. 5 C. Moreover an all-or-none action potential could be generated by an outward current pulse when the membrane potential was hyperpolarised to the level of -75 mV (Fig. 5 B).

In Na free solution containing 24 mM Ba the resting potential ranged from $+20$ mV and five out of twelve cells displayed positive values. A positive resting potential in 10 mM Ba^{2+} solution has previously been found in the rat chromaffin cell by Brailmont *et al* (1976). Fig. 5 D shows the response to anodal current break in Ba solution, and an inflexion is seen around the level of -40 mV during the repolarising phase. The inflexion may indicate the onset of a regenerative process dependent on Ba or in other words that the Ba permeability of the membrane increases when this potential level is reached. Prolonged action potentials could be evoked by outward current pulses in Ba-solution provided that the membrane potential was shifted to more negative values than -65 mV (Fig. 5 E and F). The threshold level of these action potentials coincided with the potential level where the inflexion of the repolarising phase following anodal current break was seen. This fact confirms the interpretation given above for this inflexion. The prolonged action potential often lasted more than one second (Fig. 5 F). This prolongation is probably due to a suppressive effect of Ba ions on the development of the delayed rectification, as reported for other cells (Verman and Grundfest 1961; Hagiwara and Naka 1964; Hagiwara and Kikokoro 1971).



A, spontaneous potential fluctuations in Na-free solution. Two potential components exist. B, C and D, the amplitude of both components increased with increasing intensity of the applied anodal current pulses. Further details in the text.

Spontaneous potential fluctuations

During advancement of the electrode through the pituitary slice we occasionally encountered spontaneous depolarising potentials which occurred regularly at the frequency of 4 Hz, as illustrated in Fig. 6 A. These potentials could also be seen in Na-free solution. Further inspection revealed that the potentials consist of two components. The first is a slow depolarisation on which the other faster rising and decaying potential is superimposed. The amplitude of both components increased when anodal current pulses of increasing intensity were applied through the recording electrode (Fig. 6 B-D). The records in Fig. 6 clearly show that the spike potential is preceded by a slow depolarising potential, and both components were monophasic depolarisations. In spite of this latter fact we suspect these recordings to originate from incomplete cell penetrations for the following reasons: (1) The resting potential was always less negative than -10 mV. (2) The apparent input resistance measured by shift of the bridge balance ranged from 10 to 30 MΩ, which is less than one-tenth of the value described above. (3) The potentials could not be recorded using an electrode with dull tip (resistance less than 40 MΩ), which made the slowly described intracellular recordings impossible.

The nature of these potential fluctuations is essentially similar to that reported on smooth muscle cells of the rabbit colon (Gillespie 1962) and on cat lateral geniculate neurons (McLuskin and Creutzfeldt 1967). The recordings in the latter studies were obtained by a "quasi-intracellular" condition, where the electrode was sealed into an exposed area of the membrane. The observed potential fluctuations will then reflect changes in the intensity of the current passing across the sealing resistance, and the recordings may show the true membrane potential change with some attenuation. Spontaneous potential fluctuations could also be seen in ordinary intracellular recordings from cells in Na-free solutions containing high Ca^{++} or Sr^{++} concentration, which seemed to indicate stable penetrations. Typical intracellular records from several cells superfused with

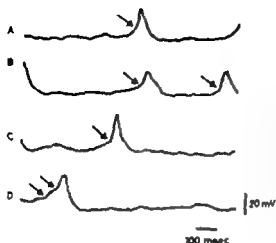


Fig. 7. Intracellular records of spontaneous action potentials in Na-free, 24 mM K^+ solution. Intracellular traces represent a continuous recording. Action potentials were initiated by small depolarisations of the membrane potential. Arrows indicate the transition between either two small depolarisations and the action potential, or between two small depolarisations (the first arrow in D). The resting potential was -35 mV and the spike generation mechanism was partially inactivated.

these solutions displayed spontaneous depolarising potentials of less than 5 mV amplitude as shown in Fig. 7. Action potentials were occasionally initiated by these small depolarisations.

Discussion

Electrical excitability

In order to see the effect of hypothalamic extracts and other secretagogues for anterior pituitary hormones on the membrane potentials of the adenohypophyseal cells, electrophysiological studies have previously been performed on the adenohypophyseal normal rats (Milligan and Kraicer 1970, Martin *et al.* 1973, York 1974). Action potentials were however never observed in these studies. On the other hand, Blakes *et al.* (1977) indicated that action potentials might occur in normal anterior pituitary cells, but no evidence was presented.

The present results clearly demonstrate that anterior pituitary cells in the normal rat are electrically excitable. The cells were electrophysiologically classified into two groups. Type I cells have action potentials with maximum rate of rise up to 40 V/s, while only regenerative responses were observed in type II cells. Type II cells had more negative stable resting potentials as well as higher input resistance than type I cells. It is therefore reasonable to assume that type II cells were less injured by the electrode penetration than type I cells, and we therefore reject the idea that the slowly rising regenerative responses are deteriorated action potentials of type I cells caused by poor penetrations. The recorded cells were not identified histologically in the present study, but the parenchymal glandular cells overwhelmingly outnumber the other cell types within the gland. Regenerative responses were seen in most of the penetrated cells, and we therefore conclude that the majority of the parenchymal glandular cells of the anterior pituitary are electrically excitable.

The fast rising action potentials of type I cells were never observed in Na-free solution.

it indicates that these action potentials are mainly due to an increase of the membrane ability to Na ions. However significant regenerative responses were almost always in Na-free solution at the termination of the inward current pulse. In Na-free solution with normal Ca^{2+} concentration both the amplitude and the rate of rise of response increased, and all-or-none action potentials could be generated by outward pulses. Action potentials could also be induced if the Ca^{2+} of this solution was reduced by ionophore Sr^{2+} or Ba^{2+} and the action potentials in all these solutions were abolished by 6 μM tetrodotoxin. From these results we conclude that the membrane of anterior pituitary cells are capable of generating Ca dependent regenerative responses (Davis 1973), and that type I cells have a Na component in addition.

The electrical properties of the normal anterior pituitary cells demonstrated in the present experiments are similar to those previously reported for clonal GH cells isolated from anterior pituitary tumour (Kidokoro 1975 Blake *et al* 1977). The present results exclude the possibility that the ability of GH cells to generate action potentials, which is partly dependent on Ca^{2+} might be caused by pathological changes of the neoplastic cells.

Physiological significance of the regenerative potentials

The regenerative responses described in the present paper usually required current injection to be evoked. It could therefore be argued that these responses might not occur under physiological conditions. However our quasi-intracellular recordings revealed spontaneous depolarising potentials. Corresponding observations were reported on GH₃ clonal cells by Kidokoro (1975) and on adrenal chromaffin cells by Brandt *et al* (1976).

In the latter cases spontaneous action potentials were rarely seen in intracellular recordings, but spontaneous extracellular spikes were recorded from nearly every cell tested using a micro-electrode. It is therefore reasonably assumed that spontaneous regenerative potentials occur in rat anterior pituitary cells under physiological conditions. In high Ca^{2+} or Sr^{2+} solution spontaneous depolarising potentials were occasionally seen even using extracellular electrodes.

It has been suggested that the Ca-dependent regenerative responses in endocrine cells are closely coupled to the hormone release. In other words, the entry of Ca^{2+} ions into the cell during an action potential may trigger the hormone release under physiological conditions (Meisner and Schmelz 1974, Kidokoro 1975 Brandt *et al* 1976 Davis and Hadley 1976). The hormone release from all the different types of secreting anterior pituitary cells requires Ca^{2+} in the external medium (Kralovic 1975). The present demonstration that Ca-dependent regenerative responses could be evoked in nearly all of the penetrated cells is consistent with the view that such responses play an important role in controlling the hormone secretion. Within the normal anterior pituitary *in vitro* only the prolactin and growth hormone secreting cells will release a significant amount of hormone in the absence of hypothalamic factors (Vale and Rivier 1975). This is in agreement with the fact that only a small number of the penetrated cells displayed spontaneous depolarising potentials, although an additional explanation of this phenomenon has been given above. Future expts. should be performed to study the effects of hypothalamic releasing and release inhibiting factors on the

Ca-dependent regenerative responses of identified anterior pituitary cells, in order to study the functional significance of these responses.

Pancreatic islet β -cells, adrenal chromaffin cells and adenohypophyseal cells have possibly a neural crest origin (Pearse 1970). Tischler *et al.* (1976) have demonstrated that all of the cultured human endocrine cells of proposed neural crest origin investigated are capable of generating all-or-none action potentials. The question then arises if only endocrine cells of neural crest origin are excitable, or in other words if electrical excitability is linked to the embryological origin of the cell rather than to its function. The present results demonstrate that even anterior pituitary cells which have not developed from the neural crest are able to generate Ca-dependent regenerative potentials.

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Blood flow distribution in the renal portal system of the intact hen. A study of a venous system using microspheres

By

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Abstract

ODLIND B. Blood flow distribution in the renal portal system of the intact hen. A study of a venous system using microspheres. Acta physiol. scand. 1978, 102, 342-356.

The blood flow in the renal portal system of intact hens was characterized by determining the fraction distribution of 15 μ m and 50 μ m microspheres in this system after injection into a leg vein. Validation showed that only 50 μ m spheres gave a reliable estimation of this distribution. The blood flow in the mesenteric vein was directed towards the liver in nearly all cases. On the average 44, 47 and 1% of portal blood from the external iliac vein perfused the ipsilateral kidney, the liver and the lung, respectively. However, the distribution of portal blood to these organs varied considerably between individuals. The distribution of portal blood to these organs changed appreciably within 35 min in half the animals studied. The reason for these variations is not clear. The portal blood from the right and left external iliac veins was asymmetrically distributed in most animals, illustrating the importance of local factors in its regulation. Variation was great in the regional distribution of portal blood within the kidney possibly due to local vasoconstriction of portal vessels. Further information on the regulation of renal portal blood flow is needed to explain its physiological significance. Microspheres provide a convenient method for such studies. A combination of microsphere and Sperry techniques allows us to determine the renal excretion efficiency of a given substance.

Key words: Portal system, microspheres, birds, kidney phlebography, venous circulation.

The avian kidney is supplied by blood from the arterial and the renal portal circulation (Sperber 1948 a, Fig. 1). The physiological significance of blood flow in different parts of the renal portal system and its regulation is not fully understood (Gilbert 1961, Silber 1971). The dual blood supply to the avian kidney has been used extensively to study renal handling of effects of drugs, hormones and auto-pharmacological agents (May and Carter 1970, Wesson 1973, Rennie 1976) utilizing a technique developed by Sperber (Sperber 1946, 1948 a). There is, however, an unpredictable variation in individual results obtained with the Sperry technique (Sperber 1948 b) which might be related to variations in blood flow in the re-

system (Akester 1964). The aim of the present investigation was to get quantitative information about the distribution of blood flow in the renal portal system, under physiological conditions and in connection with the Sperber technique. Since the microsphere technique is chosen for this purpose, it was also necessary to test the validity of the technique applied to a venous circulatory system.

Materials and Methods

Animals. White Leghorn hens, 6-18 months of age and weighing between 1.1 and 1.4 kg, from 5 different flocks and one lot of non-laying White Leghorn pullets, 4-5 months of age and weighing between 1.1 kg were used. The animals were kept on commercial feed for laying hens (Pulfor® Lantmännen, Sweden) but were fasted over night before the experiments. They had free access to tap water.

Injection procedures

Microspheres of $15 \pm 5 \mu\text{m}$ diameter (mean \pm S.D. 3 M Co. St. Paul, Minnesota, U.S.A.), labelled ^{59}Fe and 3 batches of Tracer Sephadex microspheres (Pharmacia Fine Chemicals AB, Sweden) of $15 \mu\text{m}$ diameter (mean \pm S.D.) were used. The large spheres ($50 \mu\text{m}$) were labelled with ^{57}Co or ^{54}Co by procedures recommended by the manufacturer and are carefully washed with distilled water for more than 48 h. The small spheres ($15 \mu\text{m}$) were subjected to homogenization shortly before injection in order to break up possible aggregates. The spheres were suspended in less than 1 ml of solution of Ficoll 70 (Pharmacia Fine Chemicals AB, Sweden) or in chicken plasma and were agitated slowly prior to injection. The suspensions were injected at a rate of 0.2-0.4 ml/min, followed by slow injection of 0.5 ml saline. Animals were sacrificed 5 (3-5), 25 or 60 min after the injection, using 75-100 mg/kg sodium pentobarbital injected into one brachial vein. The kidneys, the lungs and the liver were then removed. In some experiments, heart, spleen, adrenal glands, pieces of thigh muscle, some blood and parts of the intestine were also removed. The right kidney was divided into cranial, middle and caudal parts, each roughly corresponding to a division (Fig. 1) and the weight of each part was determined. The left kidney was divided into several parts. A two-channel spectrometer was used to analyse the samples. Proper calibration was present in the range of isotope activity used. The dual-isotope technique allowed simultaneous determinations of either ^{57}Co and ^{54}Co or ^{57}Co and ^{59}Fe isotopes. The hardness of the gamma-ray spectrum made correction for absorption in the samples unnecessary.

Calculation of recovery. Isotope activity exceeding 1% of the injected activity was found only in the lungs, the lungs and the liver. The sum of these activities was therefore regarded as the total recovery. The recovery of ^{59}Fe Co includes (large spheres) averaged $93.2\% \pm 3.7$ (S.D.) of the injected activity. Recovery of isotope activity as an organ was always expressed as percentage of the total recovery. Ideally these values should represent the fraction of injected microspheres trapped in the respective organs.

Calculation of the apparent splanchnic extraction fraction (ATEF)

After injection of substance into leg vein the ATEF of this substance was calculated by dividing the amount excreted in the urine on the side of the injection with the amount injected (Sperber 1948 b).

Ligation of vessels in the renal portal system

Animals. As indicated with 20-30 mg/kg sodium pentobarbital. The right kidney was exposed after flank incision. In some hens the renal anastomosis was partly freed from the ventral surface of the kidney by blunt dissection and ligated close to the bifurcation of the external iliac vein (Figures 1, Fig. 1). This ligation should prevent renal portal blood from perfusing the cranial part of the kidney and from being drained to the lungs via the cranial branch of the renal portal vein. In other animals, an additional ligation was placed around the cranial branch of the renal portal vein, here this vessel leaves the kidney (Figures 2, Fig. 1). The two ligatures should force all venous blood from the leg to perfuse the peritubular capillaries of the middle and caudal parts of the kidney (Akester 1967).

Fluorescence cinematography

In 15 hens the right kidney was exposed as described above and illuminated by 400 W metal halogen lamp (MOY Osmen GMB, München, West-Germany). Fluorescent sodium (40 solutions) was then

Blood flow distribution in the renal portal system of the intact hen. A study of a venous system using microspheres

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systems (Akster 1964). The aim of the present investigation was to get quantitative information about the distribution of blood flow in the renal portal system, under physiological conditions and in connection with the Sperber technique. Since the microsphere technique was chosen for this purpose, it was also necessary to test the validity of the technique applied to a venous circulatory system.

Materials and Methods

Animals. White Leghorns, 6-12 months of age and weighing between 1.1 and 1.4 kg, from 5 different flocks and one lot of non-laying White Leghorns pullets, 4-5 months of age and weighing between 1.1 kg, were used. The animals were kept on commercial feed for laying hens (Pulfor® Lantana, Sweden) but were fasted over night before the experiments. They had free access to tap water.

Sphere procedures

Microspheres of $15 \pm 5 \mu\text{m}$ diameter (mean \pm S.D. 3 M Co, St. Paul, Minnesota, U.S.A.), labelled 85-Sr and 3 batches of Tracer Sephadex microspheres (Pharmacia Fine Chemicals AB, Sweden) of $15 \mu\text{m}$ diameter (mean \pm S.D.) were used. The large spheres ($50 \mu\text{m}$) were labelled with 57-Co or 58-Co by procedures recommended by the manufacturer and were carefully washed with distilled water and for more than 2 weeks. The small spheres ($15 \mu\text{m}$) are subjected to homogenization shortly before use in order to break up possible aggregates. The spheres were suspended in less than 1 ml of isotonic solution of Pival 70 (Pharmacia Fine Chemicals AB, Sweden) or in chicken plasma and were agitated slowly prior to injection. The suspensions were injected at rate of 0-0.4 ml/min, followed by slow flush of 8-15 ml saline. Animals were sacrificed 5 (3-5), 25 or 60 min after the injection, using 75-100 mg/kg sodium pentobarbital injected into one brachial vein. The kidneys, the lungs and the liver were then quickly removed. The right kidney was divided into cranial, middle and caudal parts, each roughly corresponding to division (Fig. 1) and the weight of each part was determined. The left kidney was divided into several parts. A two-channel spectrometer was used to analyse the samples. Proper calibration was present in the range of isotope activity used. The dual-isotope technique allowed simultaneous determinations of either 57-Co and 58-Co or 57-Co and 85-Sr isotopes. The hardness of the gamma-rays made correction for absorption in the samples unnecessary.

Calculation of recovery. Isotope activity exceeding 1% of the injected activity as found only in the lungs, the lungs and the liver. The sum of these activities as therefore regarded as the total recovery. Recovery of Co spheres (large spheres) varied 93.2 ± 3.7 (S.D.) of the injected activity. Recovery of Sr activity in an organ was always expressed as percentage of the total recovery. Ideally these values should represent the fraction of injected microspheres trapped in the respective organ.

Calculation of the apparent tubular excretion fraction (ATEF)

After injection of substance into leg vein the ATEF of this substance was calculated by dividing the amount excreted in the urine on the side of the injection with the amount injected (Sperber 1948 b).

Ligation of vessels in the renal portal system

Anesthesia was induced with 20-30 mg/kg sodium pentobarbital. The right kidney was exposed after a flank incision. In some hens the renal anatomosis was partly freed from the ventral surface of the kidney by blunt dissection and ligated close to the bifurcation of the external iliac vein (figure 1, Fig. 1). This ligation should prevent renal portal blood from perfusing the cranial part of the kidney and from being shunted to the lungs via the two cranial trunks (Akster 1967). In other animals, an additional ligation was placed around the caudal branch of the renal portal vein, here this vessel leaves the kidney (figure 2, Fig. 1). The two ligatures should force all venous blood from the leg to perfuse the peritubular capillaries of the middle and caudal parts of the kidney (Akster 1967).

Fluorescence microscopy

In 15 hens the right kidney was exposed as described above and illuminated by 400 W metallic halogen lamp UHQY Osram GMB, München, West-Germany). Fluorescence sodium (40 solution) was then

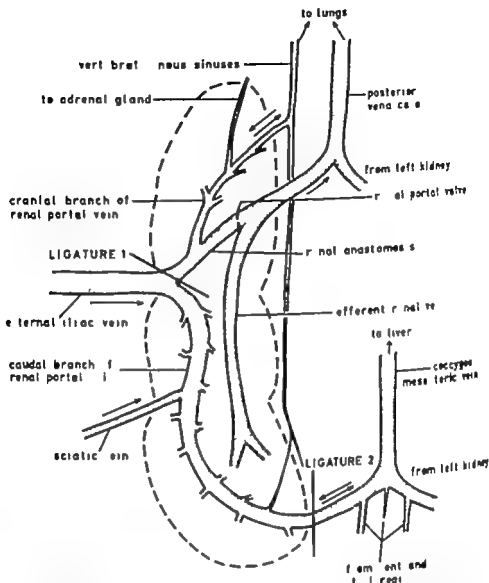


Fig. 1. Principal veins of the renal portal system of the hen (right kidney seen from the abdominal oral). Venous blood from the major part of the leg enters the ipsilateral renal portal vein (*allens lumbalis* renal vein) via the external iliac vein (*ilaca externa*). The sciatic vein (*ischiofemoralis*) conveys blood from the caudal thigh region to the caudal branch of the renal portal vein. The cranial branch of the renal portal vein communicates with the vertebral venous sinuses (Akster 1967) and with the adrenal vein system (Sperber 1948). The caudal branch of the renal portal vein communicates with the corresponding vessel from the other kidney with veins (i.e. *coccygea*) draining the vent and tail regions and with the large coccygeomesenteric vein (*coccygo-mesenterica*) draining the hind gut. The last vessel establishes a link between the renal and the hepatic portal systems. The caudal branch of the renal portal vein is also connected with the vertebral venous sinuses (Sperber 1948a, Burger and Estavillo 1977). The renal portal vein branches into the cranial, middle and caudal divisions (Goodchild 1956) of the kidney; it terminates in the peritubular (interlobular) network of capillary sinuses, where it mixes with the glomerular (arterial) blood (Sperber 1948a). Peritubular blood eventually leaves the kidney by the efferent renal vein (*renalis magna sive rectica*) which is connected to the external iliac vein by the renal anastomosis. The latter vessel has a sphincter—the renal portal valve. When this sphincter is relaxed, blood in the portal system can bypass the kidney tubules and go directly to the lungs via the posterior vena cava. Thus, there are 4 possible major afferent routes (arrows): the renal portal system and at least 3 possibilities for the blood in this system to bypass the kidney (arrows). Ligatures 1 and 2 (see text) are placed around vessels at points indicated by crossmarks.

also leg vein at the same rate as used in the microsphere injections. The kidney was observed under blue (see below) in 5 hens these events are recorded photographically by serially exposures, (2 pictures per wing Nikon F camera with MD 2 motor (Nikon Co., Japan). The Ektachrome EH 135 (Eastman Kodak Co, U.S.A.) An excitation filter (Schott BG 3) was mounted of the lamp and barrier filter (Schott OG 495) was fitted to the camera lens.

renal procedures

1960, the hens were anaesthetized and sitting in their normal perching position except following two procedures and during fluoroscopic venography when they were anaesthetized and supine. They were restrained in harness of cloth (Campbell 1960). The animals appeared to be fully conscious. Two plastic funnels were one over each ventral opening according to modification (Campbell 1960) of Sperber's original technique (Sperber 1946, 1948). Each funnel was perfused with water by an infusion pump at rate of 10 ml/min. Urine was collected in plastic vials on collection. Injections were made through polyethylene catheters (PP 25 or PP 50). Microspheres, in-heparin acid (PAH) and fluorescein sodium were injected through catheter placed in dorsal leg vein, usually the medial tarsal vein, and always on the right side, unless stated otherwise. PAH—1 mg per injection—was administered as the sodium salt (Merck Sharp and Dohme Co., U.S.A.) at doses rate of 0.6–1.4 mg/kg/min and determined as the diluted urine according to Brøn (1951).

Whether the microsphere injections had fractional effects on the kidney glomerular filtration and renal clearance of 125-I-Na-o-todobappurite (Kabi Diagnostika, Stockholm, Sweden) were noted before and after 2 doses of microspheres. For the clearance measurements, tracer doses of EDTA (ethylenediamine-tetraacetate; Behringwerke AG, Marburg, West Germany: 3×10^4 cpm/ml) 25-I-Na-o-todobappurite (6×10^4 cpm/ml) are infused through catheter placed in one brachial vein at 3 seconds, respectively 1 one then the infusion is made through catheter in leg vein on the side, which allowed ATEF to be calculated. After priming dose of 0.75 ml the infusion rate was 1 ml/min. At least 2.5 h of infusion were allowed to pass before starting the clearance determinations. Samples were drawn in the middle of each 10 min clearance period from catheter placed in vein both leg and centrifuged immediately. Lactate activity was then determined in 0.5–0.6 ml of plasma from the same samples. After 2 or 3 clearance periods during steady state, consecutive injections of 6–15 800 large spheres, at 15 min interval, were made into leg vein on the right side as described in legend to Fig. 5. Each injection, is followed by 2 or 3 clearance periods. Clearance values are calculated for each kidney separately as the mean of values from 2 or 3 collection periods. No correction for background loading of the test substances is made.

renal analysis

All test gives as the arithmetic mean \pm standard deviation (S.D.) or mean \pm standard error of the (S.E.) when appropriate. Differences in recovery of microspheres were analyzed statistically with a for paired observations (Armitage 1977) and these differences are considered significant at the 0.05 level. The orthogonal regression coefficients (Geary Scientific Tables 1970) were used to calculate the regression functions.

Results

Validation experiments

Recovery of spheres. 1 1/2 h after injection into brachial vein the recovery of small and large spheres in the lungs was 98.6% (3 hens) and 98.5% (5 hens), respectively. The escape of both types of spheres to the arterial circulation of the hen was therefore negligible, as previously reported for 15 μ m spheres (Boelkins *et al.* 1973). After injection into the coxycostocentric vein (Fig. 1) ligated caudal to the point of injection, 99.5% of the large (4 μ m) and 99.7% of the small (2 μ m) spheres were recovered in the liver 1 1/2 h post-injection. Thus both types of spheres were effectively trapped in the liver. After the simultaneous injection of both types of spheres into a leg vein of non-ligated hen, the recovery of the large spheres was on the average 1.37 ($p < 0.001$) and 0.51 ($p < 0.001$) times that of the small

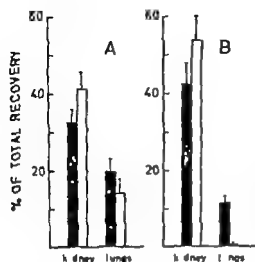


Fig. 2. Unequal distribution of small and large microspheres. Recovery of 15 μ m (■) and 50 μ m (□) splenic microspheres in the ipsilateral kidney and lungs, after splanchnic arterial anastomosis into a leg vein of A) 21 non-ligated hens and B) 11 hens with ligation of major shunts to the lungs. Values are given as mean values (columns) and 1 S.E. (error bars). Asterisks indicate statistically significant differences in mean recoveries of small and large splenic microspheres.

spheres in the *ipsilateral kidney* and *lungs*, respectively (Fig. 2 A). These differences increased with time for the three groups of hens sacrificed 5, 25 or 60 min post-injection. In the group where the renal anastomosis was ligated (ligature 1 in Fig. 1) the large spheres—in sharp contrast to the small ones ($p < 0.001$)—were almost completely prevented from reaching the lungs (Fig. 2 B). On the other hand, the fraction of large spheres trapped in the *ipsilateral kidney* of these hens was 1.28 times ($p < 0.01$) that of the value for the small ones. In the animals where the shunts both to the lungs and the liver had been blocked (ligature 2 in Fig. 1) 99.5% of the large spheres were recovered in the *ipsilateral kidney* 1 h post-injection. These findings indicate that only the large spheres were effectively trapped in the peritubular capillaries of the kidney. This is consistent with the finding that when hippuric acid (PAH) and the small spheres were injected together ATEF_{PAH} after injection was the fraction of spheres recovered in the *ipsilateral kidney* (unpublished observation). The larger spheres were consistently found in excess (see below). In view of all this the large spheres were chosen for further experiments.

Random errors of counting and distribution. The dosage of spheres administered was determined by a minimum counting precision of ± 1.0 per cent (standard deviation as percentage count) for all tissues where recovery was more than 5% of total recovery. Buck (1971) concluded theoretically and experimentally that the difference between the number of spheres in two random samples is usually less than 20% provided the number of spheres in the samples is greater than 400 and only random factors affect the distribution. In the present study 7 500 to 15 000 of the large spheres were injected and 400 microspheres per organ correspond to approximately 3 to 6% of the total recovery. The combined experimental variability was assessed (Neutze *et al.* 1968) by injecting labelled spheres simultaneously (Fig. 3). Organ recovery of ^{58}Co was taken as a reference and the deviations of the recovery of ^{57}Co from this reference was calculated as percentage. The standard deviation of these differences in recovery of the two nuclides was 5% for organs with a high mean recovery *i.e.* *ipsilateral kidney* and *liver* and 13 to 19% for organs with a low mean recovery *i.e.* *lungs* and *contralateral kidney*.

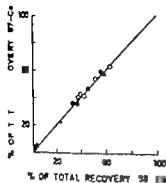


Fig. 3

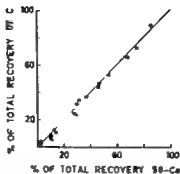


Fig. 4

Comparison of dual-isotope determinations. Recovery of ^{57}Co and ^{58}Co labelled spheres in renal kidney (\bullet), contralateral kidney (Δ), lungs ($+$) and liver (\circ) in 5 hours after simultaneous injection into leg vein. In some animals the recovery of each isotope was below 1.5% in lungs or contralateral kidney and these values were excluded. Line of identity is drawn.

Sphere distribution after separate injections. Recovery of $50\text{ }\mu\text{m}$ spheres in the ipsilateral kidney (\bullet), renal kidney (Δ), lungs ($+$) and liver (\circ) in 11 hours. Spheres, labelled with either ^{57}Co or ^{58}Co , injected simultaneously but through separate catheters, placed at different levels of one vein (-5) on the distal vein ($n=9$) of the leg. In some animals the recovery of each isotope was below 2% in contralateral kidney and these values were excluded. Line of identity is drawn. The regression lines are $y=1.14x-2.27$ and $y=1.06x-4.62$ for the ipsilateral kidney and the liver values respectively. Corresponding linear correlation coefficients (r) are -0.95 and -0.97 respectively.

of precision for the low recoveries is however of minor importance in the present study as fractional distribution was determined.

Mixing of spheres with venous blood from the leg. In 5 hens, two catheters were inserted in the same distal vein of the leg, but through separate incisions and with the tip of catheters 2 to 3 cm apart. In 6 other hens the two catheters were inserted into the medial and lateral tarsal veins, respectively. Spheres, labelled with either ^{57}Co or ^{58}Co were then injected simultaneously through the separate catheters. In both types of experiments the distribution of the two nuclides was similar (Fig. 4), indicating acceptable mixing of spheres with the blood from the lower leg.

Fluorescein injections. Injections of fluorescein sodium into the leg vein gave an intense pinkish-green fluorescence, first seen in the major portal vessels and then in the small vessels on the renal surface. After 20–30 s it had reached other organs, via the arterial circulation. At this time, the vessel walls were also fluorescent, which obscured the intraluminal fluorescence. In the early phase, however the location of the fluorescence in the portal vessels could be evaluated, especially with the rapid-sequence venography. Fluorescence was seen in the renal anastomosis in all hens, and in some cases also in the caudal branch of the renal portal vein. After most injections stream-lining of the fluorescence was seen in the major portal vessels. Location of the fluorescence within a vessel often fluctuated (within a few seconds), in some cases with the phases of respiration. This is in agreement with observations on the flow in comparable veins of several mammalian species (Hepps and McDonald 1954). However, in the present context it is important to note that there was no preferential location of the fluorescence in the external iliac vein.

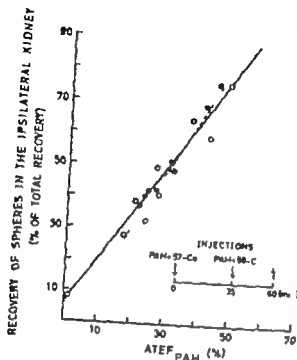


Fig. 3. Comparison of recovery of PAH ($ATEF_{PAH}$) and recovery of the ipsilateral kidney. 51-Cr and 86-Cr spheres were injected together with a leg vein of 9 hens 60 min (●) and respectively prior to sacrifice (○) (see inserted Fig. 3a). In 2 other hens an injection was made in 2 others only. The regression lines for the 60 min (solid) and the 25 min (broken) values are drawn. Linear coefficient (r) was $r = 0.96$ and $r = 0.91$ for 60 min and the 25 min values, respectively.

Comparison of techniques of microsphere recovery and PAH excretion for the estimation of portal blood flow. The relationship of ATEF for PAH ($ATEF_{PAH}$) and recovery of the ipsilateral kidney was studied by injecting labelled spheres together with PAH in a schedule as described in the legend to Fig. 3 was chosen because ipsilateral excess excretion was observed only during the first 20 min after an injection. Total recovered PAH in the urine averaged 87% after both injections. $ATEF_{PAH}$ is proportional to the fraction of venous plasma flow from the leg that perfuses the ipsilateral kidney under certain conditions are fulfilled. Firstly the peritubular plasma concentration of PAH in this study must have been well below that needed to saturate the renal transport of this substance (Tm_{PAH}; Dantzler 1966, Svendsen and Skadhauge 1976 and Odilind unpublished). Secondly the excess of PAH excreted on the side of injection must originate from extraction of the substance during first passage through the ipsilateral kidney. Glomerular filtration rate and total renal plasma flow were not determined and corrections for influence of possible asymmetries in these respects on ATEF could not be made. This contributes to the scatter of values observed in Fig. 4.

The ATEF values of PAH were found to be closely related to the recovery of spheres in the ipsilateral kidney (Fig. 5). This indicates that the spheres and PAH were similarly distributed to the peritubular capillaries of the ipsilateral kidney. The dual-isotope technique allowed this relationship to be studied at 2 different times, as described in the legend to Fig. 5.

Effect of injections of spheres on renal function. Data are given in Table I. Two consecutive injections of spheres caused no impairment of the renal clearance of 51-Cr EDTA. This implies that glomerular filtration rate was unaffected since the renal clearance of 51-Cr EDTA equals that of 3-H inulin in the hen (Odilind 1976). There were no statistically

Effects of 10 consecutive injections of spheres on renal function. For details see Experimental procedures. Clearance values (mean values in ml/min/kg) for ^{51}Cr EDTA (C_{EDTA} 5 hens) and ^{125}I -Na-o-iodohippurate (C_{IHP} 3 hens) and ATEF values of the latter (ATEF_{IHP} 1 hen) are shown. The paired differences between ipsilateral and contralateral clearance values are given as mean \pm S.D. Recovery of spheres in the ipsilateral kidney was 42-83 %.

	ipsilateral kidney	contralateral kidney	difference (ips-const)	
C_{EDTA}	2.12	2.07	0.05 ± 0.14	
C_{IHP}	2.05	2.13	-0.08 ± 0.09	
C_{IHP}	2.11	2.13	-0.02 ± 0.07	
				ATEF _{IHP}
C_{EDTA}	19.6	18.3	1.3 ± 0.8	44.9
C_{IHP}	17.1	18.5	-1.4 ± 0.8	41.3
C_{IHP}	16.9	18.5	-1.6 ± 1.1	40.1

10 injections of spheres. In another hen the two injections of spheres caused no change in ATEF values of continuously infused ^{125}I -Na-o-iodohippurate. These findings suggest that injections of spheres had no effect on the tubular secretion of the acid. Furthermore, no change in total renal plasma flow could not have occurred without a change in C_{EDTA} . The ratio of ATEF_{IHP} to recovery of spheres in the ipsilateral kidney (calculated from data in Fig. 5) was similar ($p < 0.05$) after the first ($60.1 \pm 4.5\%$ mean \pm S.D.) and the second ($65.1 \pm 6.9\%$) injection. This shows that the excretion efficiency of PAH was unaltered by a preceding injection of spheres.

Distribution of spheres in different tissues

Renal distribution. Fig. 6 shows the fractional distribution of spheres in 76 intact hens of five different lots and in 13 hens prepared for urine collection according to Sperber. A high recovery in one organ of a given animal, is accompanied by a correspondingly lower uptake in the other organs. Recoveries were determined at 5 or 25 or/and 60 min after the injection. Whether there was any redistribution of the spheres. In intact as well as prepared hens there were no statistical differences in mean recovery of spheres in the respective organs at the three time intervals post-injection. This indicates that redistribution of spheres (50 μm) during the first hour after the injection was negligible.

Also, there was no statistical difference in mean recovery of spheres between intact and prepared hens which indicates that the latter procedure did not cause any important changes in renal blood flow distribution compared to the intact animal.

Taking all animals together (102 injections in 89 animals) the recovery of spheres in the ipsilateral kidney averaged $43.5 \pm 17.8\%$ (S.D.). Hepatic recovery averaged $47.3 \pm 20.6\%$ (S.D.) and was less than 5% in only 5% of the injections. Pulmonary recovery averaged 5% and exceeded 10% in less than 22% of the injections. Recovery in the contralateral kidney averaged 1.5% and exceeded 5% in only 6% of the injections. The inter-individual variation in tissue distribution was extensive also within a single lot, as exemplified by Fig. 7. *Ureter distribution within the ipsilateral kidney.* Spheres were unequally distributed in the two parts of the kidney. In 84 hens the distribution of spheres within the ipsilateral kidney

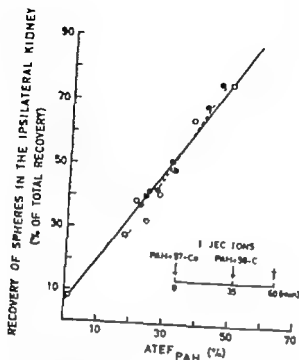
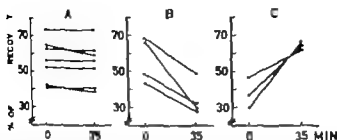


Fig. 5 Comparison of tubular PAH ($ATEF_{PAH}$) and recovery of the ipsilateral kidney. 57-Co and 58 spheres were injected together in a leg vein of 9 hens 60 min (●) and respectively prior to sacrifice (○) (see inserted Fig.). In 2 other hens a injection was made, in 2 others only one. The regression lines for the 6 (1.48 + 5.20·solid) and the 25 min (○ broken) values are drawn. Linear coefficient (●) was $r=0.96$ and $r=0.60$ min and the 15 min values, respect

Comparison of techniques of microsphere recovery and PAH excretion for the estimation of portal blood flow. The relationship of ATEF for PAH ($ATEF_{PAH}$) and recovery of the ipsilateral kidney was studied by injecting labelled spheres together with PAH, schedule as described in the legend to Fig. 5 was chosen because ipsilateral excess excretion was observed only during the first 20 min after an injection. Total renal PAH in the urine averaged 87% after both injections. $ATEF_{PAH}$ is proportional to fraction of venous plasma flow from the leg that perfuses the ipsilateral kidney to certain conditions are fulfilled. Firstly the peritubular plasma concentration of PAH in this study must have been well below that needed to saturate the renal transport of this substance (Tm_{PAH}, Dantzler 1966, Svendsen and Skadhauge 1976 and Odell 1976). Secondly the excess of PAH excreted on the side of injection must originate from extraction of the substance during first passage through the ipsilateral kidney. Glomerular filtration rate and total renal plasma flow were not determined and corrections for influence of possible asymmetries in these respects on ATEF could not be made. The scatter of values observed in Fig. 5.

The ATEF values of PAH were found to be closely related to the recovery of spheres in the ipsilateral kidney (Fig. 5). This indicates that the spheres and PAH were similarly distributed to the peritubular capillaries of the ipsilateral kidney. The dual-isotope technique allowed this relationship to be studied at 2 different times, as described in the legend to Fig. 5.

Effect of injections of spheres on renal function. Data are given in Table I. Two consecutive injections of spheres caused no impairment of the renal clearance of 51-Cr-EDTA, implying that glomerular filtration rate was unaffected since the renal clearance of 51-EDTA equals that of 3-H-inulin in the hen (Odlind 1976). There were no statistically significant changes in bilateral renal clearances of 125-I Na-O-40 (C_{125I}) (Odlind 1976).



A-C Change in renal uptake of spheres with time. Recovery of 30 μ m spheres in the ipsilateral after two consecutive injections at 35 min interval, using the dual-isotope technique. The spheres passed through catheter into leg vein of the right leg of 14 hens. Results are given in three groups: changed, B) decreased and C) increased renal uptake of spheres. Note broken scale on the ordinate, to connect the two observations of each animal.

high, however uptake in the caudal part of the kidney dominated, and only a few were found in the cranial part. A similar skew distribution within the kidney was seen for radiopaque fluid when injected into a leg vein (unpublished observations).

Asymmetric distribution after bilateral injection. Using the dual-isotope technique, spheres were injected simultaneously into a vein of the right and left legs, respectively. In most hens, as well as non-laying, spheres injected on the right side showed a different organ distribution compared to spheres injected on the left side. This is illustrated in Fig. 7 for the series in the ipsilateral kidney and the liver but was also found for the recoveries in the β . For spheres injected on the right side (18 hens), the average recovery was 26.2% lower (8.6%) in the kidney and 26.4% higher ($p < 0.05$) in the liver compared to the corresponding results for spheres injected on the left side. Laying and non-laying hens did not differ in this respect. The average recovery in the lungs was the same regardless of the side of injection.

Change of distribution with time. These experiments were designed to see if the distribution of spheres to the organs changed with time or as the result of a previous injection. Two suspensions of differently labelled spheres were injected with a 35 min interval (as described in legend to Fig. 5). There was no difference in the mean recoveries of the two nuclides; in the ipsilateral kidney it was $49.9 \pm 4.1\%$ (S.E.) and $49.0 \pm 3.2\%$ (S.E., $p > 0.8$) after the first and the second injection, respectively in 18 animals. (From data in Fig. 8 A-C.) Thus, there was no clear effect of the first injection on the distribution after the second injection. However the distribution of spheres after 2 injections could vary considerably in the individual hen, as is shown in Fig. 8 B and C for the ipsilateral renal recovery in seven hens. In 17 other hens the two nuclides were equally distributed (Fig. 8 A).

Discussion

Validity of the microspheres technique in the study of the renal portal circulation

The 15 μ m microspheres could not be used, since they were incompletely trapped in the renal capillaries of the kidney (Fig. 2). In contrast to the almost complete pulmonary

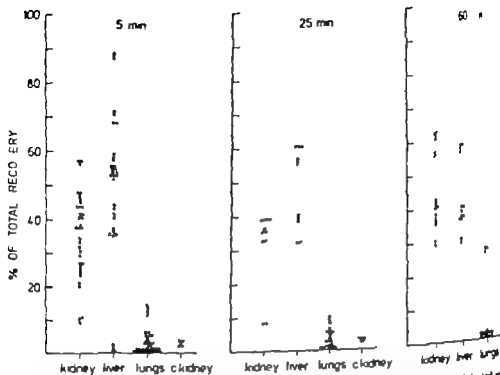


Fig. 6. Distribution of spheres in different organs. Organ recoveries of 50 μ m spheres injected in the right leg in 76 intact hens (\bullet) at 5 ($n=46$), 25 ($n=15$) or 60 min ($n=15$) respectively and in 12 hens prepared for urine collection according to Sperber (\circ) at 25 and 60 min, post-injection. Values for the contralateral kidney (c. kidney) are not shown. L. kidney stand for ipsilateral kidney. The no. of injections are given.

(mean \pm S.E.) was $14.2 \pm 1.6\%$, $40.9 \pm 1.9\%$ and $45.0 \pm 2.1\%$ in the cranial, mid and caudal parts, respectively. The weight (mean \pm S.E.) of these parts were 2.49; 2.21 ± 0.08 g and 3.11 ± 0.11 g, respectively for 48 kidneys. In animals with a low recovery the spheres were evenly distributed between the three parts. When hepatic

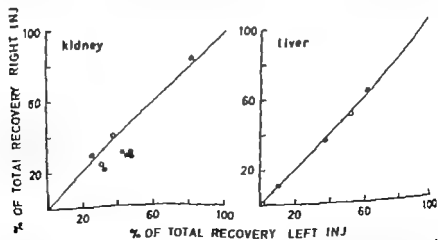


Fig. 7. Asymmetric distribution of spheres after bilateral section. Recovery of 50 μ m spheres in kidney and liver after simultaneous injection in veins of the right (ordinates) and the left (abscissae) of 12 laying hens of the same lot (\bullet) and 6 non-laying (\circ) hens of different lots, using the double technique. Asterisks indicate mean values. Lines of identity are drawn.

Direction of blood flow in the renal portal system

Blood flow in the coccygeomesenteric vein was nearly always directed towards the liver and there were spheres in this organ after 95% of the injections. This observation is supported by the high and persistent recovery of spheres in the caudal part of the kidney. A flow in the opposite direction of the liver is in agreement with the findings of Sturkie and Abati (1975), as well as non-fasting birds, using electromagnetic flowmeter probes. Other workers have reported a blood flow in both directions between the two portal systems (Spanner, Clarkson and Richards 1966, Akester 1967, Purton 1970, 1975). These studies, however, include different experimental procedures such as anaesthesia, supine position or abdominal surgery which could have affected the hemodynamic situation in the portal system, as pointed out by Sperber (1948 a). Results presented in the present paper are conscious hens in a normal body position, and are therefore presumably more representative of conditions in the intact animal.

It is more difficult to estimate the direction of flow in the most cranial shunt (Fig. 1), where the recovery of spheres in this part of the kidney was low. The small size of this shunt, however, makes a large contribution by "backward" flow from the vertebral sinus accessible (Akester 1967). Regional differences in the intrarenal distribution of spheres are due to local vasoconstriction of major portal vessels in the kidney as proposed by Purton (1967). That local vasoconstriction can occur in the renal portal system is suggested by radiographic studies on the renal localization of 35-S-furosemide when injected into the caudal vein. The distribution of this diuretic was often restricted to certain regions, leaving other areas unlabelled (Dencker and Odling 1977). A nervous regulation of blood flow in the renal portal system is implied by the dense, predominantly adrenergic innervation of the renal portal system (Benoist and Malenfort 1970).

Summary: it is obvious that in the unanesthetized hen the bulk of the blood in the renal portal system is supplied by the external iliac and the sciatic veins. The blood from the sciatic vein could either have perfused the caudal part of the kidney or could have been directed along the coccygeomesenteric vein to the liver. Spheres were not injected into the sciatic vein since it is inaccessible to external catheterisation. However, due to the larger size of the external iliac vein the fractional distribution of blood flow from this vessel should be representative of the renal portal system as a whole.

Regional distribution of blood flow in the renal portal system

The present study demonstrates a striking variation in the distribution of renal portal blood between individuals (Fig. 6), within the kidney between right and left side (Fig. 7) and between the two portal systems (Fig. 8).

Taken together these results reveal a very complex flow pattern in agreement with Akester's (1964, 1967) findings in anesthetized birds using a radiographic (qualitative) technique. Several general conclusions are of interest. On the average, the kidney received less than half of the renal portal blood from the leg, and in no case did it receive all of this blood. Thus, a shunt of portal blood bypassing the kidney tissue, was always present. In nearly all hens the coccygeomesenteric vein was the dominant shunt vessel. This fits with the high rate of blood flow in this vessel as reported by Sturkie and Abati (1976). One could deduce from these

and hepatic extraction of these spheres. This difference suggests a "leakage" of some of the small spheres initially deposited in the kidney and may be a consequence of the unusual structure of the peritubular capillaries in the renal cortex of birds (Sperber 1944 a). Extensive pulmonary extraction of both types of spheres excludes recirculation of any importance. It is crucial, when using microspheres to assess blood flow distribution, that the spheres are adequately mixed with the blood and distributed to various organs in proportion to the blood flow. In the arterial circulation mixing can be accomplished by injecting the spheres into the left heart ventricle (Hales 1974) and can be verified by comparing a) the sphere concentration in paired organs, *i.e.* kidneys (Neutze *et al.* 1968, Boelkins *et al.* 1973) or b) the concentration of spheres in peripheral arteries (Neutze *et al.* 1968, Hoffbrand and Forry 1968). In analogy with these results, in the present study spheres reaching the right ventricle were found to be equally distributed between the two lungs ($y = -0.08 + 1.06 X$, linear correlation coefficient $r = 0.98$). However mixing conditions in the peripheral venous circulation are not easily evaluated. Nolan *et al.* (1975) found nonhomogenous distribution within the liver of colloidal $\text{Cr}^{51}\text{PO}_4$ or $15 \mu\text{m}$ resin microspheres after injection into the portal vein of rats, in contrast to the homogenous dispersion in the liver of $\text{Cr}^{51}\text{PO}_4$ injected into an external artery. The difference was attributed to stream-lining of the particles in the blood at portal injection. In the present study the spheres were injected into a distal vein at a slow rate, which resulted in reasonably even mixing of the spheres with the blood from the lower leg (Fig. 4). However the application of spheres to the study of the venous circulation presents another validation problem since venous blood is continuously being added by vessels proximal to the site of injection. This could result in a preferential peripheral streaming of the spheres in the main vein and hence would lead to a systematic distributional error at bifurcations. It was therefore essential to see if this occurred at the most critical point, which is in the external iliac vein. It was not possible to visualize microspheres labelled with fluorescein-isothiocyanate in the larger vessels, possibly due to axial streaming (Philbbs and Doolittle 1970) and coating of the spheres with erythrocytes (Philbbs *et al.* 1967). However there was no preferential streaming of fluorescein in the external iliac vein and it seems likely that PAH, a substance of comparable molecular weight, would be handled similarly. Since it was shown that the spheres and PAH were similarly distributed to the peritubular capillaries of the ipsilateral kidney (Fig. 5) it seems reasonable to exclude preferential peripheral streaming of spheres in the major portal vessels as an important source of error in the overall distribution. Possible distributional errors due to fluctuations between axial and peripheral streamings were minimized by making the injections slowly for a period of 2-4 min.

It seems unlikely that gravitational forces could influence the distribution of spheres since the relative density of Tracer Sephadex (large) spheres is close to that of red blood cells (1.12 g/ml and 1.10 g/ml respectively). From the validation experiments with ϕ microspheres, it can be concluded that the technique used, gives a reliable estimation of the fractional distribution of blood flow from the external iliac vein to the kidneys, the liver and the liver.

the coccygeomesenteric vein and therefore also the hepatic portal system are parts, reduces the role often ascribed to the renal portal valve as the dominant regulatory of portal blood flow to the kidney (Rennick and Ghandia 1954, Gilbert 1961). The sphere technique provides a valuable tool for further studies of the regulation of renal blood flow in the hen.

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authors' findings that fasting affects the distribution of renal portal blood flow. The possibility remains, however, to be tested. Spheres terminating in the lungs could have bypassed the kidney via one or both of the cranial shunts (Fig. 1) and the technique used could not discriminate between these routes. However, the renal portal valve must have been open or nearly closed in the majority of cases (Fig. 6). Contrary to these findings in intact birds, the most common shunting route in supine and anesthetized birds was that through the renal portal valve (Akester 1967). It is obvious, that the renal portal valve is only one of several factors involved in the regulation of renal portal blood flow.

Some further points are worthy of note. In most animals, blood flow from the right and left external iliac veins was differently distributed (Fig. 7). This is in agreement with findings that radiopaque fluid took different routes through right or left renal portal system after bilateral injections in about 25% of the birds (Akester 1967).

The reason for a higher average flow to the kidney from the left external iliac vein compared to the right one (Fig. 7) is not clear. The difference was not due to the large size of the oviduct attached to the left kidney since it was seen in laying as well as non-laying hens.

Duplicate determinations, at 35 min interval, revealed a considerable change in flow pattern from the leg in 50% of the hens, in spite of constant experimental and environmental conditions. There was a covariation in these cases mainly between the renal and the femoral fractions. The physiological basis for this "intrinsic" variation with time is not clear. In future studies, very rapid (within seconds) vascular readjustments, related to the renal portal valve, have been described by Akester (1964). Interestingly, the valve has a dense autonomic innervation (Gilbert 1961; Akester and Mann 1969; Dolezel and Zidek 1969; Bennet and Malmfors 1970) which has been studied *in vitro* by Rennick and Gilman (1954) and by Bennet and Malmfors (1975).

The fraction of portal out of total blood flow to the kidney is difficult to measure directly. It has been suggested to be 2/3 (Sperber 1960) or 1/2 (Skadhauge 1973) in conscious birds. Ratios calculated from indirect observations. Assuming a relatively constant venous return from the resting leg, it may be expected that the large intra- and interindividual variation in the renal fraction of portal blood described in the present paper reflect a variation in renal perfusion rate. This would lead to highly variable renal portal to total blood flow ratios and is of considerable interest since it provides a possible mechanism by which total peritubular blood flow can change irrespective of the arterial blood flow to the kidney. In this connection, it is worthy of note that Sapirstein and Hartman (1959) found a considerably higher arterial perfusion rate of leg muscles than of pectoral muscles in the anesthetized bird, providing a high venous return from the leg and thus to the renal portal system.

It is evident (Fig. 5 and 7) that the individual results obtained with the Sperber technique will vary mainly with the renal portal perfusion fraction. A combination of the microsphere and the Sperber techniques, however, enables a correction for the influence of "shunting" of renal portal blood on the excretion (ATEF) values. This will also allow an estimation of the renal excretion efficiency that is the true tubular excretion fraction (Sperber 1968b) of a given substance.

In sum, it seems that changes in the blood flow distribution in the renal portal system are the effect of hemodynamic readjustments (sometimes rapid) in a vascular network, in

Effects of pindolol, sotalol and the optical isomers of propranolol
on muscle action potentials and depolarization-secretion
coupling in the rat

By

ANDRÉO LARSEN

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Abstract

REX, A. Effects of pindolol, sotalol and the optical isomers of propranolol on muscle action potentials and depolarization-secretion coupling in the rat Acta physiol. scand. 1978 102, 357-363.

Muscle action potentials and miniature end-plate potential frequency were studied in different concentrations of pindolol, *d*- and *l*-propranolol and sotalol using intracellular microelectrode recording from rat muscle nerve-diaphragm preparations. *d*- and *l*-propranolol at concentrations of 10 to 40 mg/l and pindolol 20 to 100 mg/l slowed down the rise of rise, prolonged rise and half-decay times and reduced the overall of the muscle action potentials. Sotalol had similar effects but only at higher concentrations (100 to 8 mg/l). The drugs had no effect on the increase in the miniature end-plate potential frequency obtained on depolarization by increased extracellular potassium concentration.

Key words: Optical isomers of propranolol, pindolol, sotalol, action potentials, miniature end-plate potentials, skeletal muscle, microelectrode recording.

Pindolol, *d*- and *l*-propranolol and sotalol have been shown to possess curare-like postsynaptic effects at the neuromuscular junction (Liljeheil and Roed 1971, Wermann and Wiklöf 1971, Larsen and Terkivilinen 1977, Larsen 1977). Pindolol and both optical isomers of propranolol also have presynaptic actions. They depress stimulus-induced release of transmitter quanta from the motor nerve endings (Larsen and Terkivilinen 1977, Larsen 1977). Since this effect manifested itself rapidly and was reversible, two alternative mechanisms remained to explain it. A reduction of the probability of release following a depolarization of the motor nerve endings, and a reduction of the presynaptic depolarization in the form of a diminished amplitude of the nerve action potential are both possible.

The present study was undertaken in order to investigate these alternatives by studying the effects of pindolol, sotalol and the isomers of propranolol on the depolarization-induced increase in transmitter release and on muscle action potentials. The effect on muscle action potentials is regarded as a model of their membrane effect and is presumed to correlate to the presynaptic action potential.

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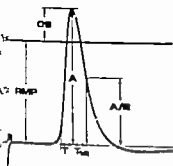


Fig. 1 The variables measured from the action potentials. A = amplitude, OS = overshoot, T = rise time, $T_{1/2}$ = half-decay time. The rate of rise is calculated as A/T . The line in the recording indicates the zero potential, RMP is the resting membrane potential and S is the stimulus artifact.

propranolol was observed to cause qualitatively and quantitatively similar effects as *l*-propranolol on the configuration of the APs, the mean values obtained for the two isomers being less than 2% from each other at all different concentrations tested. For this reason values of the two isomers are not shown separately in Fig. 3 but represented by their common mean value at each concentration.

Sotalol and sotalol had effects qualitatively similar to those of *d*- and *l*-propranolol on the configuration of the action potentials, but higher concentrations were needed (Fig. 3). The concentrations needed to cause a 20% change from the control value in the variables measured from the action potentials were 4 to 7 times higher for pindolol and 30 to 50 times higher for sotalol than for either *d*- or *l*-propranolol. At the different concentrations tested, the effect of *d*- and *l*-propranolol (10, 20 and 40 mg/l) and pindolol (20, 60 and 100 mg/l) on the variables measured was statistically highly significant ($p < 0.001$) compared with the control values. Sotalol, at a concentration of 40 mg/l, prolonged the half-decay time and reduced the overshoot significantly ($p < 0.01$). Highly significant ($p < 0.001$) changes in the half-decay time and the overshoot of the action potentials were observed at a concentration of 100 mg/l. Concentrations of sotalol up to 100 mg/l did not have significant effects on the rise time and rate of rise of the action potentials, but the rise time was prolonged and the rate of rise slowed down highly significantly ($p < 0.001$) at 200 mg/l (Fig. 3).

There were no significant differences in the miniature end-plate potential (MEPP) frequency at fibres treated with *d*- or *l*-propranolol or sotalol compared with the control values in solutions containing either 5 mmol/l or 20 mmol/l potassium (Table 1), but the MEPP fre-

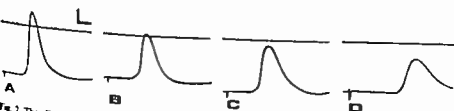


Fig. 2 The effect of different concentrations of *l*-propranolol on the action potentials. A is control, B, C and D are recorded in solutions containing *l*-propranolol 10, 20 and 40 mg/l, respectively. The line indicates the zero potential. Scale: 20 mV and 1 ms. The overshoot of the action potentials is reduced, rise time and half-decay time prolonged and the rate of rise slowed down by *l*-propranolol.

Methods

Altogether 44 Sprague-Dawley rats weighing 190 to 250 g were used. The animals were decapitated under ether anaesthesia, and the left hemidiaphragm muscle with the phrenic nerve was removed and prepared further in continuously oxygenated bathing solution. The preparations were then placed in a recording chamber with a fluid capacity of 4 ml, the solution being replaced at a rate of 400 ml per hour. The bathing solution had the following composition (in mmol/l): NaCl 135, NaHCO₃ 16.5, Na₂HPO₄ 1, KCl 1, CaCl₂ 2, MgCl₂ 1 and glucose 11. It was equilibrated with a mixture of 95% O₂ and 5% CO₂, and had a pH of 7.35–7.40. A solution containing 20 mmol/l of KCl and 120 mmol/l of NaCl was used to study the increase in miniature end-plate potential frequency in response to depolarization of the nerve cells (Liley 1956, Takeuchi and Takeuchi 1961). The drug to be studied was dissolved in the bathing solution and introduced to the bath from another reservoir. The drugs used were pindolol base (Sandoz Ltd, Sweden) mixed with tartaric acid 2:1 *d*- and *l*-propranolol hydrochloride (ICI Pharmacia OY, Finland) or sotalol hydrochloride (Läike OY, Finland). The preparations were equilibrated in the drug-free solution in the solutions containing one of the drugs for 30 min before the recording was begun and after the change of solution. All the recordings were made at a bath temperature of 23°C.

Resting membrane potentials (RMP), miniature end-plate potentials (MEPP) and action potentials (AP) were recorded intracellularly with glass capillary microelectrodes filled with 3 M KCl and sealed to resistances between 5 and 15 megohms. The potentials were fed through a Mentor N-990 preamplifier and displayed on a Tektronix 5115 storage oscilloscope. The traces were photographed from the oscilloscope screen and measured from magnified negatives. Only those fibres with an initial RMP of at least 60 mV negative inside and which did not depolarize more than 5 mV during the recordings were used. The microelectrode penetration was made under stereomicroscope control about 6 mm away from the end-plate regions when recording APs, and as near as possible to the transverse nerve when MEPPs were recorded. The criterion for focal placement of the microelectrode to the end-plate region was the recording of MEPPs with rise time shorter than 1 ms (Nickels 1970).

The MEPP frequencies were calculated first from 10 fibres in a solution containing 5 mmol/l potassium and then from another 10 fibres in a solution containing 20 mmol/l potassium (high K⁺ solution) in each preparation. At least 100 MEPPs were counted from each fibre. The MEPP frequency ratio (MFR) was calculated as $MFR = (\text{mean MEPP frequency in high K solution}) / (\text{mean MEPP frequency in 5 mmol/l K solution})$ in each of the preparations. Each of the drugs was tested in 4 different preparations and 16 preparations were studied without any drugs.

Muscle action potentials were studied after stimulating the phrenic nerve through suction electrode with supramaximal current pulses 0.02 ms in duration. The pulses were generated by Grass S8 stimulator with an STU 4678 stimulus isolation unit. Only one AP was recorded from each fibre because of frequent fibre damage and microelectrode replacement due to contraction of the fibres. The variables chosen to illustrate the effects of the drugs on the APs are: rise time (T_r), half-decay time (T_{1/2}), overshoot and rate of rise of the AP as defined in Fig. 1. Each concentration of each drug was tested in 2 different preparations. Control values were taken before the drug was added.

Student's *t*-test was used to determine the statistical significance of the differences between the means.

Results

None of the drugs studied caused significant differences in the resting membrane potential recorded in normal bathing solution containing 5 mmol/l potassium. The resting membrane potential recorded without any drug from 85 fibres was 75.8 ± 7.2 mV (mean \pm S.D.), and that of 70 fibres recorded with pindolol was 74.7 ± 6.9 mV. The corresponding figures were 76.9 ± 7.5 mV for 70 fibres recorded with *d*-propranolol, 74.9 ± 8.0 mV for 70 fibres with *l*-propranolol and 77.1 ± 7.9 mV for 70 fibres in sotalol.

The effect of different concentrations of *l*-propranolol on the muscle action potentials is shown in Fig. 2. *l*-Propranolol caused a dose-dependent reduction of the overshoot of the action potential, as well as a prolongation of rise and half-decay times and a slowing down of the rate of rise (Fig. 2 and 3).

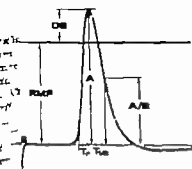


Fig. 1 The variables measured from the action potentials. A = amplitude, OS = overshoot, T_r = rise time, T_d = half-decay time. The rate of rise is calculated as A/T_r . The line in the recording indicates the zero potential, RMP is the resting membrane potential and S is the stimulus artifact.

propranolol was observed to cause qualitatively and quantitatively similar effects as *l*-propranolol on the configuration of the APs, the mean values obtained for the two isomers being less than 2% from each other at all different concentrations tested. For this reason the shapes of the two isomers are not shown separately in Fig. 3, but represented by their common mean value at each concentration.

Sotalol and sotalol had effects qualitatively similar to those of *d*- and *l*-propranolol on the configuration of the action potentials, but higher concentrations were needed (Fig. 3). The concentrations needed to cause a 20% change from the control value in the variables measured from the action potentials were 4 to 7 times higher for pindolol and 30 to 90 times higher for sotalol than for either *d*- or *l*-propranolol. At the different concentrations tested, the effect of *d*- and *l*-propranolol (10, 20 and 40 mg/l) and pindolol (20, 60 and 100 mg/l) on the variables measured was statistically highly significant ($p < 0.001$) compared with the control values. Sotalol, at a concentration of 40 mg/l, prolonged the half-decay time and reduced the overshoot significantly ($p = 0.01$). Highly significant ($p < 0.001$) changes in the half-decay time and the overshoot of the action potentials were observed at a concentration of 100 mg/l. Concentrations of sotalol up to 100 mg/l did not have significant effects on the rise time and rate of rise of the action potentials, but the rise time was prolonged and the rate of rise slowed down highly significantly ($p < 0.001$) at 200 mg/l (Fig. 3).

There were no significant differences in the miniature end-plate potential (MEPP) frequency in fibres treated with *d*- or *l*-propranolol or sotalol compared with the control values in solutions containing either 5 mmol/l or 20 mmol/l potassium (Table 1), but the MEPP fre-

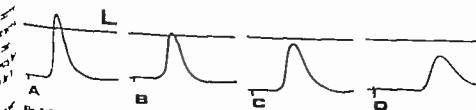


Fig. 2 The effect of different concentrations of *l*-propranolol on the action potentials. A = control. B, C and D are recorded in solutions containing *l*-propranolol 10, 20 and 40 mg/l, respectively. The line indicates the zero potential. Scale: 20 mV and 1 ms. The overshoot of the action potentials is reduced, rise time and half-decay time prolonged and the rate of rise slowed down by *l*-propranolol.

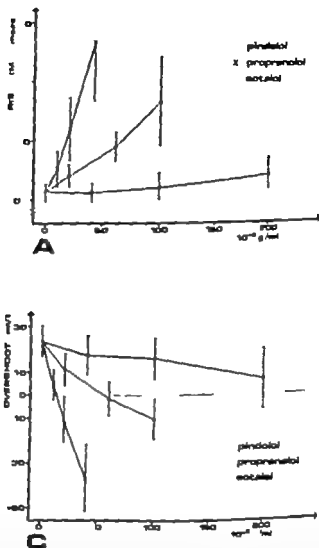
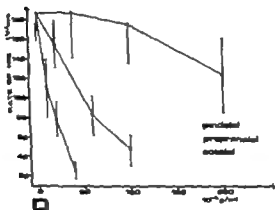
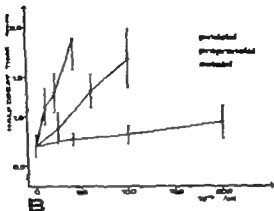


Fig. 3. The effect of pindolol, sotalol and the optical isomers of propranolol on the action potential rise time is shown in relation to their concentration. A shows the effect of the drugs on the rise time, B on the decay time, C on the overshoot and D on the rate of rise of the action potentials. Abscissa: The concentration of the drug. The signs indicate mean and S.D. those of control being calculated from 60 fibres. Those of pindolol and sotalol from 30 fibres each. The values of *d*- and *l*-propranolol differed less than 10% from each other at all concentrations tested, and are therefore represented by their common lines, each being calculated from a total of 60 fibres, 30 treated with *d*-propranolol and 30 with *l*-propranolol.

frequencies in pindolol containing solutions were higher ($p < 0.01$). The MEPP frequency in high K⁺ solution (20 mmol/l) increased about 32 to 36-fold (Table I) compared with control. With 5 mmol/l potassium, this increase being of about the same magnitude with all the drugs. The depolarization of the muscle fibre membrane, caused by the high extracellular potassium concentration, was of the same magnitude in all the experiments (Table I).

Discussion

All the drugs tested reduced the overshoot, prolonged the rise and half-decay times and slowed down the rate of rise of skeletal muscle fibre action potentials. *d*- and *l*-propranolol

β -BLOCKERS ON MUSCLE ACTION POTENTIALS

propranolol had the strongest effect on a weight basis, whereas the effect of sotalol was weak, seen first at concentrations 30 to 50 times higher than those of propranolol. None of the drugs affected the increase in miniature endplate potential frequency in response to depolarization caused by increased extracellular potassium concentration.

The effects of these drugs on the action potentials of skeletal muscle fibres resemble their effects on the cardiac muscle action potentials. In the cardiac muscle fibres 1 to 3 mg/l of propranolol and 6 to 10 mg/l pindolol reduce the amplitude overshoot, decrease the rate of rise and prolong the rise time (Morales-Aguilera and Vaughan Williams 1965, Davis and Joseph 1968, Singh and Vaughan Williams 1971, Maruyama 1973). These effects are also seen with sotalol, but at higher concentrations, 50 mg/l and over (Singh and Vaughan Williams 1970, Scrimgeour *et al.* 1970).

The mechanism by which the drugs affect the muscle fibre action potentials is probably interference with the ionic conductances of the muscle fibre membrane. Because the drugs were observed to prolong the rise time and to reduce the overshoot of the action potentials, it is likely that they block the action potential.

TABLE 1 The effect of pindolol, sotalol and the optical isomers of propranolol on the increase in miniature end-plate potential (MEPP) frequency produced by a high concentration of potassium. Each value represents the mean \pm S.E. of 4 preparations. MEPP frequency ratios (MFR) have been calculated as (mean MEPP frequency in 20 mmol/l K)/(mean MEPP frequency in 5 mmol/l K) in each of the preparations. RMP is the resting membrane potential.

	MEPP fr In 5 mmol/l K (s ⁻¹)	MEPP fr In 20 mmol/l K (s ⁻¹)	MFR	RMP in 20 mmol/l K (mV)
Control	1.6 \pm 0.29	57.9 \pm 5.4	36.2 \pm 4.2	56.7 \pm 4.3
<i>d</i> -Propranolol (20 mg/l)	1.59 \pm 0.30	56.3 \pm 5.9	35.5 \pm 4.6	54.9 \pm 4.9
<i>l</i> -Propranolol (20 mg/l)	1.61 \pm 0.27	55.0 \pm 5.1	34.7 \pm 4.9	55.9 \pm 5.3
Pindolol (40 mg/l)	4.8 \pm 0.36	81.1 \pm 11.1	32.9 \pm 4.2	46.1 \pm 4.8
Sotalol (40 mg/l)	1.56 \pm 0.31	55.9 \pm 7.2	35.8 \pm 6.3	55.2 \pm 5.0

Significant ($p < 0.01$) difference compared with the control.

1952). The prolongation of the half-decay time may reflect an effect on the potassium channels, but it may also result from a delayed inactivation of sodium channels during repolarization (Hodgkin and Huxley 1952). In frog atrial muscle *d*- and *l*-propranolol suppress the late inward sodium current of action potentials, without affecting the potassium current (Larssen *et al.* 1973). In squid axons *d*-propranolol blocks the impulse conduction by interfering predominantly with the sodium conductance but in addition suppresses also the potassium conductance (Wu 1972, Wu and Narahashi 1973).

Previously it has been shown that both *d*- and *l*-propranolol and pindolol, but not sotalol, have presynaptic effects by diminishing the number of acetylcholine quanta released per nerve impulse (Larsen and Teräsväinen 1977, Larsen 1977). This presynaptic action of pindolol and the both isomers of propranolol is probably due to the "membrane effect" similar to that in the muscle fibres, i.e. an interference with the ionic conductance of the excitable membranes. On the basis of their effect on the muscle action potentials, it can be expected that pindolol and *d*- and *l*-propranolol also reduce the amplitude of the presynaptic nerve action potentials and thus diminish the presynaptic depolarization caused by a nerve impulse. This leads to a reduced number of transmitter quanta released (Katz and Miledi 1967). A reduction in the probability of release is not likely because the drugs did not decrease the depolarization-induced increase in the miniature end-plate potential frequency.

Sotalol has previously been reported to have postsynaptic but not presynaptic effects (Larsen 1977). On the basis of the present observations, sotalol may however also have presynaptic effects but only at considerably higher concentrations than those needed to produce postsynaptic effects.

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Urinary cyclic AMP and vasopressin excretion in rat strains selected for their alcohol intake

By

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Abstract

LINKOLA J and F FYHRQVIST *Urinary cyclic AMP and vasopressin excretion in rat strains selected for their alcohol intake* Acta physiol. scand. 1978. 102. 364-367

Urinary excretion of adenosine 3',5'-cyclic monophosphate (cAMP) and immunoreactive arginine vasopressin (AVP) were investigated after water loading and following ethanol loading in two rat strains selected for their voluntary ethanol intake. After ethanol loading ethanol preferring (AA) rats excreted more cAMP but less AVP than water preferring (ANA) rats. The results suggest that the strain difference in cAMP excretion is of renal origin and is not due to vasopressin or parathormone. Differences in the sympathetic nervous activity may be responsible for the difference in cAMP excretion.

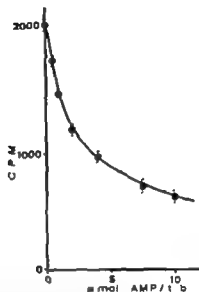
Two rat strains (AA = Alko Alcohol and ANA = Alko Non-Alcohol) have been selected for their voluntary ethanol drinking (Eriksson 1968, Eriksson 1971). Diuretic response to ethanol was lower in alcohol preferring (AA) rats than water preferring (ANA) rats (Linkola *et al.* 1977a). In accord with this, AA rats excreted more vasopressin into urine than ANA rats (Linkola *et al.* 1977a), when ethanol was administered to non-hydrated rats. However, when ethanol was administered to prehydrated rats, the strain difference in urine output was opposite (Linkola 1976). AA rats excreted more urine in that situation.

In this study urinary excretion of AVP was studied during ethanol diuresis in prehydrated AA and ANA rats. Because of the crucial role of cAMP as a cellular mediator of many hormones, among them AVP (Douza and Valtin 1976) the urinary excretion of cAMP was also measured.

Material and Methods

10 four-month old, male AA and ANA rats of the F₁₀ generation, weighing 208-363 g, were used in the experiment. They were fed and loaded with water and ethanol as described earlier (Linkola 1974), except that 20% v/v ethanol was administered 2.4 g/kg b.wt., and urine was collected into plastic tubes in ice bath.

Urine AVP was determined by radioimmunoassay (Fyhrqvist *et al.* 1976, Linkola *et al.* 1977a) and urine cAMP by protein binding assay as follows. Fresh bovine adrenals were used as a source of binding protein. They were homogenized in 50 mM Tris/HCl buffer containing 4 mM Na₂EDTA, pH 7.5 (EDTA buffer) as described (Linkola *et al.* 1977b). After acetic acid and ammonium sulphate steps described in



A standard curve for cAMP. Each point represents 4 determinations \pm S.D. CPM = counts per minute.

As detailed by Alames and Sövik (1978), protein preparations as dissolved in the EDTA buffer and centrifuged against the same buffer. The dialyzed solution was centrifuged at 15 000 r.p.m. for 30 min. The separation of the binding protein were performed below $+4^{\circ}\text{C}$. After adjustment of the buffer to contain 12 $\mu\text{g}/\text{ml}$ of the EDTA buffer the binding protein as stored in portions of 1 ml at -20°C . For standard cAMP assay 1 ml of the binding protein was diluted with 9 ml of the EDTA buffer. The samples were diluted 1:50 in the EDTA buffer which was also used as an assay buffer. Other assay conditions were as described (Linkola *et al* 1977b), except 50 μl of diluted urine was used. A typical standard curve for cAMP is presented in Fig. 1. For statistical evaluation Student's t-test was employed.

Results

Water output. During water loading ANA rats excreted more urine than AA rats (Table I). When ethanol was administered there was no strain difference in urine output during the first hour but during the following two hours AA rats excreted more urine than ANA rats, the difference was not statistically significant.

AVP output. Urinary excretion of immunoreactive AVP was more pronounced in AA rats than ANA rats during the water loading period (Table I). The difference was, however, not statistically significant. After ethanol loading ANA rats excreted about three times more AVP than AA rats during the first hour but no significant difference was observed during the following 2 h.

CAMP output. There was no significant difference in urinary excretion of cAMP during water loading period. After ethanol loading AA rats excreted about twice as much cAMP as ANA rats during the first hour (Table I). The same difference continued during the two following hours.

Discussion

Urine output. Higher urine output in ANA rats than AA rats after water loading confirms previous findings (Linkola 1976, Linkola *et al* 1977). Similarly support is given to the

TABLE I Urine output (ml/kg h), urinary excretion of AVP (pg/kg h) and cAMP (nmol/kg h) during water loading (8-11 a.m.) and after ethanol loading (11-12 a.m. and 0-2 p.m.) in AA (n=10) and ANA (n=10) rats. Means \pm S.D. p means the significance of the strain difference.

	AA	ANA	
Urine output			
8-11 a.m.	17.0 \pm 4.3	2.5 \pm 1.7	p < 0.005
11-12 a.m.	20.9 \pm 2.9	20.8 \pm 5.8	
0-2 p.m.	7.1 \pm 3.1	5.1 \pm 1.5	
AVP output			
8-11 a.m.	138.1 \pm 114.0	89.3 \pm 105.1	
11-12 a.m.	95.8 \pm 90.0	301.9 \pm 184.4	p < 0.01
0-2 p.m.	118.9 \pm 71.6	117.1 \pm 44.4	
cAMP output			
8-11 a.m.	13.3 \pm 3.9	1.3 \pm 4.1	
11-12 a.m.	19.9 \pm 8.5	8.5 \pm 4.2	p < 0.005
0-2 p.m.	18.1 \pm 3.0	9.4 \pm 3.3	p < 0.001

earlier report (Linkola 1976) that in prehydrated rats ethanol causes stronger diuresis in AA rats than ANA rats. That the strain difference was less obvious than earlier may be due to different prehydration procedure or to differences between rat generations. An earlier report (Linkola 1976) was made with F₁ rats, while F₃₆ rats were used in this work.

AVP output Because urine was collected during one and two hours, possible fluctuations in AVP excretion within short intervals could not be estimated. Higher AVP output in AA rats than ANA rats after water loading agrees with previous results (Linkola 1976; *et al.* 1977a). The remarkable increase in AVP output in ANA rats after ethanol loading was not seen in AA rats, may partly explain the diuresis of AA and ANA rats (Table I; Linkola 1976) because it has been reported that urinary AVP output reflects the plasma concentration of this hormone (Miller and Moses 1971).

The reasons for strong AVP output in ANA rats as compared to AA rats after ethanol loading remain unclear. AVP release may be suppressed by noradrenaline (Berl *et al.* 1974) in AA rats. As suggested (Linkola 1976) it is possible that the primary cause for the difference in the diuresis between AA and ANA rats lies not in the release of AVP but, more in the autonomic nervous system affecting AVP release.

Sodium ions may also contribute to AVP release in ANA rats after ethanol loading because more sodium is apparently accumulated in prehydrated, ethanol loaded ANA rats than in AA rats (Linkola 1976). Conversely the retention of sodium may also be a consequence of the actions of AVP in the kidneys (Hornych *et al.* 1973).

cAMP output The difference in the urinary excretion of cAMP may be at the level of cAMP synthesis or at the level of the factors stimulating cAMP synthesis. Among the factors are vasopressin (Dousa and Valtin 1976), parathormone (Chase and Aurba 1976) and catecholamines (Dousa and Valtin 1976).

Vasopressin is not likely to be responsible for the strain difference in cAMP output because ANA rats excreted more AVP after ethanol loading than AA rats. Neither AVP values alone explain the diuretic response of the rats after water and ethanol loading (Table I). cAMP output indicates that other cAMP mediated processes may also contribute to urine output of the rats.

There is no data on parathormone of AA and ANA rats. However unpublished results after plasma calcium concentration and higher urinary calcium excretion of ANA rats suggest that parathormone was not responsible for the strain difference in IP excretion.

Our unpublished observations showed no higher plasma cAMP concentration in AA rats than in ANA rats, it is likely that the strain difference in cAMP excretion is of J origin. Ethanol stimulates adenylyl cyclase in many tissues (Vollicer and Gold 1975). However the slight strain difference in cAMP excretion already during water loading indicates that ethanol is not needed to cause difference in cAMP excretion. Catecholamines also stimulate adenylyl cyclase in the kidneys (Dousa and Valtin 1976, Beck *et al.* 1972), resulting in release of renin. Our recent unpublished observations indicating higher plasma renin activity in AA rats than ANA rats therefore suggest that the actions of catecholamines in kidneys may be responsible for the strain difference in cAMP excretion.

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Estrogen and the relaxant effect of intramural noradrenaline on calcium induced contractures in depolarized rat uterus

By

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Abstract

BENOTSSON B. *Estrogen and the relaxant effect of intramural noradrenaline on calcium induced contractures in depolarized rat uterus.* Acta physiol. scand. 1978. 102. 368-373.

The influence of intramural noradrenaline on calcium induced contractures was studied in isolated preparations of rat uterus. The depolarized (1.7 mM KCl) myometrium of oophorectomized rats responded to contraction followed by a transient relaxation when exposed to 3 mM calcium. The threshold concentration of calcium, where the transient relaxation began to appear was between 0.25 and 0.5 mM. Blockade of the β -adrenoceptors with propranolol or noradrenaline depletion with reserpine completely removed the transient relaxation, indicating that the latter was due to release of intramural noradrenaline. Estrogen treatment abolished the relaxant effect of intramural noradrenaline whereas progesterone had no effect in this respect. Preparations from rats in natural estrus responded like estrogenized tissues, and decidual preparations behaved as uteri of oophorectomized rats without estrogen treatment.

The isolated rat uterus, depolarized in an isotonic potassium solution, produces graded contractures in response to calcium added to the external medium (Edman and Schill 1962). The present study showed that the calcium contracture under certain conditions displayed a quite complex pattern. Thus, when the influence of estrogen was small, the contractile response was found to contain 3 distinct phases: an initial rapid rise of tension, a partial relaxation, and a subsequent slow increase of tension. The different phases of the contraction could possibly be explained on the basis of different sources of activator calcium, for instance one with rapid and another with slow transport of calcium into the contractile system. Quite another possibility is that calcium releases a biologically active substance stored in the uterus, which causes a transient relaxation and thereby interrupts the otherwise smooth tension rise.

The present paper describes experiments designed to further elucidate the mechanisms behind the calcium contracture of depolarized rat uterus. Results show that the transient relaxation occurring during the calcium contracture is caused by released intramural noradrenaline. A preliminary report on this investigation was given previously (Benotsson 1978).

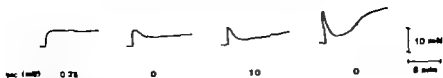


Fig. 1. Contractile responses to different concentrations of calcium in a depolarized (127 mM K) uterus from oophorectomized rat. Calcium concentrations as indicated in the figure.

Material and methods

thirty oophorectomized and 10 normal virgin rats (150–200 g) of the Wistar strain. 10 of the operated animals received 0.5 mg polycystradiol phosphate and 4 were given 5 mg oxyprogesterone caproate as an intramuscular injection immediately after operation. The remaining oophorectomized animals received no hormone treatment and served as controls. They were killed on day 7 after operation. 30 h before the experiment, reserpine (5 mg/kg) was given to 5 of the controls and 5 of the oophorectomized rats. In rabbits, the treatment depletes the uterus of noradrenaline within 24–36 h (Owman and Sjoholm 1967).

In the 36 rats with intact ovaries, 5 were in early oestrus and the rest in dioestrus when used in the experiments. The oestrus cycle was determined by histological examination of the vaginal smears. After operation and oestrogen treatment the rats were killed and quickly eviscerated. The uterus was isolated, cleaned of blood vessels (see below), and split open by longitudinal sections at the mesenteric borders of the uterus. The uterine horns were divided transversely into pieces about 15 mm long. These were mounted singly in 30 ml isolated organ baths and stretched to a tension of 10 mN, which gradually reduced to a basal tension of 1–2 mN. After 10 min the muscular tension was recorded isometrically by Grass 20-C preamplifier connected to Grass P7 polygraph ink-writer.

Experimental procedure. The uterine strips were allowed to equilibrate for 30 min in ordinary Krebs solution. During this time, regular spontaneous contractions appeared in all preparations except those from oophorectomized animals. In the latter case, only infrequent contractions occurred (cf. Lissner *et al.* 1971). Preparations were contracted by immersion in potassium-Krebs solution (see below) containing 1.5 mM calcium. After 15 min, the preparations were relaxed by immersion in calcium-free potassium-Krebs solution. The muscles were kept in the calcium-free medium for 30 min before new contractions were evoked by addition of calcium chloride into the bathing solution (final conc. 3 mM). In one series of experiments, the muscles were evoked with calcium in different concentrations (0.25–4.0 mM). Between the contractions, the muscles were bathed in calcium-free potassium-Krebs solution for 15 min.

Solutions. The muscle strips were bathed in Krebs solution, composition (mM): NaCl 119, KCl 4.6, $NaHCO_3$ 20, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11, pH 7.4. The calcium-free potassium-Krebs solution had the following composition (mM): KCl 127, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, EGTA 0.01, glucose 11, pH 7.4. The solutions were prepared on the day of the experiment. Double-distilled water and analytical grade chemicals were used.

The bathing solutions were kept at 37°C and aerated with a mixture of 5% CO_2 and 95% O_2 . Drugs: Reserpine (BDH), oestradiol-17 β (BDH), oxyprogesterone caproate (Progeston-Depot[®] Schering), polycystradiol phosphate (Estradormin, Leo), proprenolol (Indanal[®] ICI-Pharm), and reserpine (Leyland[®] Ciba).

Drugs were injected into the back in volumes of 0.03–0.1 ml, 55 min before the calcium contractions were evoked.

Results

Calcium contractions in strips of oophorectomized rats

Fig. 1 shows a sequence of contractile responses in a depolarized uterine strip subjected to increasing concentrations of calcium. At 0.25 mM calcium, the tension rose smoothly to a level which then was maintained. Calcium at higher concentrations (0.5–4 mM) did not evoke contractions which directly reached a sustained maximum, but evoked a phasic

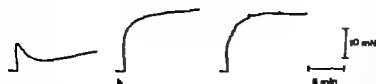


Fig. 2. Contractile responses to 3 mM calcium in depolarized rat uterine strips from oophorectomized rat: (a) control, (b) in the presence of propranolol (2×10^{-6} M), (c) after reserpinization.

contraction followed by relaxation. The active tension, after reaching a minimum, slowly rose to a sustained level. The initial rise and the subsequent decline of tension became steeper as higher concentrations of calcium were used (cf. Fig. 1 b and d), and with myoelectric stimulation the duration of the relaxation phase became shorter.

The results shown in Fig. 1 suggested that the complex pattern of the calcium contracture was based on a relaxing substance released from stores in the uterus when calcium was added to the medium. Further evidence in support of this idea was provided by the experiments in Fig. 2. A recording of a control response to 3 mM calcium demonstrates a typical transient relaxation of a depolarized uterus (Fig. 2 a). Another strip of the same uterus, incubated with propranolol (2×10^{-6} M) in order to block the β -adrenoceptors of the myometrium, responded to calcium with a smoothly developing contracture, without transient relaxation (Fig. 2 b). The calcium contracture in myometrium of reserpinized rat (see Methods) is shown for comparison (Fig. 2 c). Depletion of neuronal noradrenaline and β -adrenoceptor blockade had identical effects, indicating that the transient relaxation of the calcium contracture in the depolarized rat uterus was due to the release of intramural noradrenaline.

Effects of estrogen and progesterone on calcium contractures. Isolated uteri of rats treated with estrogen (see Methods) produced calcium contractures without transient relaxation (Fig. 3 a) similar to non-estrogenized uteri treated with sympatholytic agents (Fig. 2 b, d). As a result neither propranolol nor reserpinization changed the calcium contracture of myometrium from estrogen-treated rats (Fig. 3 b, c).

It might be argued that after estrogen treatment intramural acetylcholine was released in amounts sufficient to compensate for released noradrenaline. However, as Fig. 3 d shows, the calcium contracture was not changed by blockade of the muscarinic receptors of the myometrium by atropine (10^{-5} M). Lack of influence of atropine on the calcium contracture was also noted in non-estrogenized myometrium.

In order to find out whether estrogen exerted an immediate effect on the contractile response to calcium, myometrium from non-estrogenized (and oophorectomized) animals was incubated with $3 \mu\text{M}$ estradiol- 17β for 55 min before calcium was added to the depolarizing solution. However, *in-vitro* treatment with estrogen did not prevent the transient decline of tension during the calcium contracture (Fig. 4 a).



Fig. 3. Contractile responses to 3 mM calcium in depolarized myometrium from estrogen-treated rats: (a) control, (b) in the presence of 2×10^{-6} M propranolol, (c) after reserpinization, (d) in the presence of 10^{-5} M atropine.

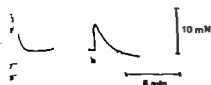


Fig. 4



Fig. 5

Fig. 4 Contractile responses to 3 mM calcium in depolarized uterine strips from oophorectomized rat. (a) with 3×10^{-6} M nitroglycerin in the bathing solution, (b) injected with 5 mg oestrogen before the experiment.

Fig. 5 Contractile responses to 3 mM Ca in depolarized uterine strips from unoperated rats in () diestrus (a) estrus.

Nitroglycerin given to the animal for 4 days before the expt. had no obvious effect on the calcium contracture, which occurred similar to that of control preparations (Fig. 4 b).

Calcium contractures in *Meri f* rats in intact ovarian function

The contractile response to calcium in the depolarized rat uterus varied with the animal's oestrous cycle. Thus the uterus of diestrus rats exhibited a transient relaxation of the calcium contracture in very much the same way as uteri of oophorectomized rats (Fig. 5 a), whereas preparations from rats in early estrus responded with a smooth rise in tension i.e. similar to the response observed in estrogenized preparations (Fig. 5 b).

Discussion

The rat uterus receives both parasympathetic and sympathetic nerves (Adham and Schenk 1967), and as calcium has the ability to activate the mechanism causing transmitter release from nerve endings (for review see Rubin 1970, Kirpekar 1975), a release of both noradrenaline and acetylcholine is likely to occur when a preparation of the rat uterus is exposed to calcium. Both these substances have been shown to influence the contractility of depolarized rat myometrium, noradrenaline reduces active tension (Schäld 1967), whereas acetylcholine induces contraction (Evans *et al.* 1959).

As atropine had no effect on the contractile response to calcium in the present study the amount of acetylcholine released can be assumed to be insignificant (*cf* Border 1940, Des and Pickford 1964).

Noradrenaline, on the contrary was evidently released in sufficient amounts to counteract the contractile effect of calcium in the non-estrogenized uterus, as the calcium contracture in this tissue as augmented by both reserpization and β -adrenoceptor blockade. The threshold concentration of calcium where the transient relaxation began to appear indicating release of noradrenaline, was between 0.25 and 0.5 mM (Fig. 1). It is of interest to note that the release of acetylcholine from cholinergic motor nerves is considerably increased when the concentration of extracellular calcium is raised above 0.2 mM (Dodge and Rahamimoff 1967).

The adrenergic influence on the calcium contracture was removed by treatment with oestrogen. This is partially explained by two effects of estrogen in the rat uterus: 1) the amount

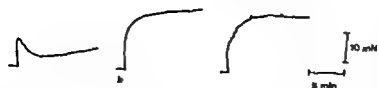


Fig. 2. Contractile responses to 3 mM calcium in depolarized strips from oophorectomized rats: (a) control, (b) in the presence of 2×10^{-6} M propranolol, (c) after reserpine treatment, (d) in the presence of 2×10^{-6} M reserpine.

contraction followed by relaxation. The active tension, after reaching a maximum, rose to a sustained level. The initial rise and the subsequent decline of tension became steeper as higher concentrations of calcium were used (*cf.* Fig. 1 b and d), and with repetitive stimulation the duration of the relaxation phase became shorter.

The results shown in Fig. 1 suggested that the complex pattern of the calcium response was based on a relaxing substance released from stores in the uterus when calcium was added to the medium. Further evidence in support of this idea was provided by the experiments in Fig. 2. A recording of a control response to 3 mM calcium demonstrates a typical rise and relaxation of a depolarized uterus (Fig. 2 a). Another strip of the same uterus, incubated with propranolol (2×10^{-6} M) in order to block the β -adrenoceptors of the myometrium, responded to calcium with a smoothly developing contracture, without transient relaxation (Fig. 2 b). The calcium contracture in myometrium of reserpine-treated rats (see Method) was shown for comparison (Fig. 2 c). Depletion of neuronal noradrenaline and β -adrenoceptor blockade had identical effects, indicating that the transient relaxation of the calcium contracture in the depolarized rat uterus was due to the release of intramural noradrenaline.

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In order to find out whether estrogen exerted an immediate effect on the contractile response to calcium, myometrium from non-estrogenized (and oophorectomized) rats was incubated with 1μ M estradiol 17β for 55 min before calcium was added to the depolarizing solution. However, *in-vitro* treatment with estrogen did not prevent the transient decline of tension during the calcium contracture (Fig. 4 a).



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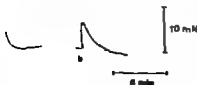


Fig. 4



Fig. 5

4. Contractile responses to 3 mM calcium in depolarized uterine strips from oophorectomized rats with 10^{-4} M atropine-173 in the bathing solution, (b) injected with 5 mg oxyprogesterone caproate 48 h before the experiment.

5. Contractile responses to 3 mM Ca in depolarized uterine strips from oophorectomized rats in () diestrus (b) estrus.

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The adrenergic influence on the calcium contracture was removed by treatment with phenoxybenzamine. This is partially explained by two effects of estrogen in the rat uterus. 1. the amount



Fig. 2. Contractile responses to 3 mM calcium in depolarized strips from oophorectomized rats. (a) control, (b) in the presence of $2 \cdot 10^{-6}$ M propranolol, (c) after reserpine treatment.

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of noradrenaline is decreased per weight smooth muscle tissue (Oskarsson 1960, Fald 1974) 2. the sensitivity to the relaxant effect of noradrenaline is reduced (Bengtsson 1977a) However even the combined effect of decreased concentration and reduced sensitivity is probably insufficient to explain the complete elimination of the influence of intramural noradrenaline shown in this study As will be described in a coming paper estrogen also causes the release of H noradrenaline in the rat uterus (Bengtsson 1977 b).

Estrogen did not remove the transient relaxation of the calcium contracture when added to the uterus *in vitro* However a number of physiological effects of estrogen require favourable conditions to develop and have not been reproduced *in vitro* (for review see Spatzman 1977)

The myometrium of rats in natural estrus responded to calcium in a way similar to that of estrogen treated animals, whereas the diestrus preparations behaved like the oophorectomized rats without estrogen treatment This is understandable, as the estrus phase is preceded by the maximum estrogen secretion of the estrus cycle, and as diestrus occurs after 2-3 days of minute estrogen secretion (Yoshinaga *et al* 1969 Brown-Grant *et al* 1970, Shaikh 1971) The present finding that the relaxant effect of intramural noradrenaline is absent during estrus agrees with an earlier report of Deis and Pickford (1969) Investigating contractions of the rat uterus *in situ* they found that the contractile activity of the diestrus uterus was greater when the sympathetic nerves were blocked, suggesting active adrenergic inhibition at this time In contrast, in estrus uterine contractions were unchanged during sympathetic blockade, indicating that inhibitory sympathetic input was eliminated during this phase

Progesterone, contrary to estrogen had no sympatholytic action in the present experiments In fact given together with estrogen, progesterone would probably exert an indirect pathomimetic effect, as gestagens, possibly by reducing the number of estrogen receptors, have an antiestrogenic effect in the uterus (for review see Brenner and West 1973).

I wish to thank Professor K. A. P. Edman for advice and helpful criticism during the course of the investigation, Professor K. E. Andersson for helpful discussions and criticism of the manuscript and Ms Christina Olsson for skilful technical assistance.

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the heart, simultaneous inhibition of α - and β -receptors was required to abolish the PGE release caused by NA (Wennumalm 1975).

In the present study the PGE release following infusion of NA and other sympathomimetic amines was studied in the isolated rabbit heart in both the absence and the presence of adrenergic drugs. The aim was to find out whether sympathomimetic drugs, when releasing PGE, act directly on some type of adrenoceptive mechanism in the tissue, or indirectly inducing e.g. contraction of the organ.

Methods

Technique of heart perfusion

Hearts of adult strains and sexes were used. The weight of the animals varied from 1.4 to 2.5 kg. They were killed by blow on the head and exsanguinated by cutting the left carotid artery. The hearts were quickly removed and transferred to the perfusion apparatus, where it was perfused according to Langendorff with Tyrode solution of the following composition (in mM): Na^+ 149, K^+ 2.7, Ca^{2+} 1.8, Mg^{2+} 1.0, H_2PO_4^- 12, PO_4^{3-} 0.4, glucose 5.5. The solution was continuously aerated with 5% CO_2 in O_2 and pH of 7.4-7.5. The perfusion pressure was 80 cm H_2O and the temperature 37°C. The apex of the heart was connected to a strain gauge transducer and heart rate and contractile force were recorded on a Model 5 II Polygraph. The heart rate recording was repeatedly calibrated during the course of the experiment. The strain gauge transducer was loaded in the load range of 0-5 pounds, and thus permitted the qualitative recording of changes in contractile force during the experiment. All experiments were started after the heart had begun spontaneously beating in the perfusion apparatus for 10-15 min. During the experiments the effluent from the organ was collected in consecutive 10 min periods for 40 min.

The sympathomimetic drug to be infused was administered with an infusion pump connected to a catheter in the aorta. The infusion rate was chosen to produce a final concentration of the drug of 10^{-6} M. After experiments the α -adrenoceptor inhibitor phentolamine or the β -adrenoceptor inhibitor propranolol was added to the Tyrode solution from the beginning of the experiment, at a concentration of 5×10^{-6} M. Drug infusion was performed during the second 10 min effluent collection period. The first, third and fourth effluent collection periods served as controls, and the mechanical activity and the PGE overflow as well as the coronary flow rate during these periods were regarded as basal.

Analysis of prostaglandins

The effluent from the heart was acidified, and its contents of lipids was extracted twice with equal amounts of ethyl acetate. After washing of the organic phase to neutral reaction, it was evaporated to dryness, and the residue was dissolved in a small amount of solvent. The PGE-like activity of the dissolved residues was tested against known amounts of PGE₂ on the rat stomach strip (Vane 1957), incubated in an organ bath with 1.5 ml volume, containing 37°C Tyrode solution of the composition mentioned above. To the solution was added phentolamine (7×10^{-6} M), propranolol (8×10^{-6} M), atropine (10^{-6} M), acetylcholine (6×10^{-6} M) and dihydroergocristine (7×10^{-6} M), in order to block activity on the strip due to the presence in the effluent of NA, A, acetylcholine, 5-hydroxytryptamine or histamine.

The sensitivity of the assay organ was accepted when 5 ng of PGE₂ induced definite and reproducible contractions. As a consequence an outflow rate of PGE from the heart corresponding to 1 μg PGE₂/hour could be accurately measured. Each lipid sample was tested twice, the intra-individual variation usually being less than 15%. The recovery of PGE was regularly checked by the use of external standards of PGE₂ and was found to range between 80 and 90%. The values given for outflow of prostaglandins are expressed in PGE₂ equivalents and are not corrected for losses during the purification procedure. For identification of PGE in the effluents from the hearts two extracts were subjected to thin layer chromatography (Green and Lamounier 1964).

Statistical analysis

Values in the text, tables and figures are presented as means \pm S.E. of means. When brackets indicate number of experiments. For estimation of the change in prostaglandin outflow caused by drug infusion the rates before and during the infusion period and the mean outflow during the preceding and following period were calculated. Student's *t*-test for paired differences has been used when applicable.

Prostaglandin mediated inhibition of noradrenaline release
IV Prostaglandin synthesis is stimulated by myocardial
adrenoceptors differing from the α and β -type

By

Å WENNMALM and T BRUNDIN

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Abstract

WENNMALM Å. and T BRUNDIN *Prostaglandin-mediated inhibition of noradrenaline release*
IV Prostaglandin synthesis is stimulated by myocardial adrenoceptors differing from the
 α and β -type Acta physiol. scand. 1978. 102. 374-381

The release of prostaglandin E elicited by sympathomimetic amines was studied in the isolated rabbit heart. The hearts were prepared according to Langendorff with conventional recording of stroke frequency and contractile force. Assays were made of the outflow of PGE during exposition to equimolar concentrations of methoxamine, noradrenaline, adrenaline and isoprenaline in the absence and in the presence of phentolamine or propranolol. Noradrenaline caused an almost four-fold increase in the basal outflow of PGE from the heart, while methoxamine (an α -adrenoceptor agonist) and isoprenaline (a β -adrenoceptor agonist) were both ineffective in this respect. Thus, the PGE-releasing capacity of the drugs is not correlated to their ability to activate α or β -adrenoceptors. Furthermore, no relation was obtained between the PGE release induced by the drugs and the increase in heart rate and contractile force elicited by them. It is suggested that sympathomimetic drugs trigger PGE synthesis and release in the rabbit myocardium following activation of a hitherto unobserved adrenoceptive mechanism, optimally stimulated by NA.

Sympathetic nerve stimulation has been shown to cause release of prostaglandin E (PGE) from different tissues and species, e.g. the rat epididymal fat pad (Shaw 1966), the dog spleen (Davies *et al* 1967, Gilmore *et al* 1968) and the rabbit heart (Wenmalm and Stjärne 1971). Since noradrenaline (NA) (and adrenaline (A) as well) upon infusion have been shown to cause PGE release (Gilmore *et al* 1968, Junstad and Wenmalm 1973) the factor triggering the release is probably the neurotransmitter liberated.

While it has thus been demonstrated in different tissues and species that adrenoceptor agonists may release PGE from sympathetically innervated organs the type of receptor mechanism underlying this release is incompletely known. In the dog spleen the PGE release (and the mechanical response) caused by A failed to materialise at all upon pretreatment with phenoxybenzamine (Davies *et al* 1967), indicating that α -adrenoceptor activation and contraction of the organ might be involved in the release of PGE. perfused

11.5. Chronotropic and inotropic responses to infusion of sympathomimetic amines at 10^{-6} M in the absence or presence of adrenergic agents. The chronotropic response is expressed as the maximal percent increase in heart rate during drug infusion compared to the heart rate prior to the infusion. The inotropic response is expressed as the maximal recorded increase in contractile force during the drug infusion compared to the mean contractile force before and after the infusion.

(a)	Positive chronotropic response	Positive inotropic response
Noradrenaline	0 (3)	0 (3)
Phenolamine		
no adrenergic drug present	33 ± 9 (4)	63 ± 14 (4)
1 phenolamine	37 ± 1 (4)	0 (4)
5 10^{-6} present	0 (4)	30 ± 9 (4)
propranolol	0 (4)	0 (4)
5 10^{-6} present	0 (4)	0 (4)
Adrenaline		
no adrenergic drug present	66 ± 8 (5)	69 ± 9 (5)
1 phenolamine	39 ± 4 (7)	30 ± 12 (7)
5 10^{-6} present	0 (10)	63 ± 8 (10)
propranolol	0 (10)	0 (10)
5 10^{-6} present	73 ± 12 (4)	22 ± 8 (6)

Noradrenaline

Infusion of NA induced a marked increase in heart rate and contractile force. Omission of the drug infusion was followed by restitution of the basal heart rate, while the contractile force failed somewhat. During the infusion of NA an almost 4-fold increase ($P < 0.01$) in the outflow of PGE was observed, which also returned towards the basal level after the end of the NA infusion.

In the presence of the α -adrenoceptor blocker phenolamine the frequency response of the heart to NA was unaffected, while the contractile response disappeared completely. The NA induced overflow of PGE was almost abolished in the presence of phenolamine (Fig. 2).

Following β -blockade with propranolol no frequency response to NA was obtained, while the inotropic response remained almost unchanged. The overflow of PGF induced by NA infusion was slightly but insignificantly reduced.

Adrenaline

Infusion of adrenaline elicited a pronounced chronotropic and inotropic response. The outflow of PGE was markedly ($P < 0.001$) increased, by $160 \pm 40\%$ (Fig. 1).

Phenolamine did not significantly affect the chronotropic or inotropic responses to A. The PGE overflow observed during A infusion was markedly reduced only an insignificant reduction was noted when A was infused in the presence of phenolamine.

TABLE I Prostaglandin outflow expressed in ng/min, from the perfused rabbit heart during 4 consecutive 10 min periods. The respective sympathomimetic drugs were infused during the second 10 min period (10-20) at a rate producing a final concentration of 10^{-6} M. Outflow of prostaglandins significantly differing from the mean outflow during the preceding and the following period indicated by: * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Perfusion conditions	I	II	III	IV
	0-10	10-20	20-30	30-40
1 Methoxamine	3.4 ± 1.0 (5)	$2.0 \pm 0.7^*$ (5)	3.0 ± 0.8 (5)	2.1 ± 0.5 (5)
2 Noradrenaline				
a. no adrenolytic drug present	2.0 ± 0.4 (4)	11.0 ± 2.1 (4)	—	4.8 ± 1.7 (4)
b. phentolamine $5 \cdot 10^{-6}$ M present	3.3 ± 0.7 (4)	7.0 ± 0.8 (4)	—	3.1 ± 0.8 (4)
c. propranolol	3.4 ± 0.5 (4)	2.0 ± 0.7 (4)	—	2.6 ± 0.6 (4)
3 Adrenaline				
a. no adrenolytic drug present	3.4 ± 1.0 (5)	7.5 ± 0.8 (5)	—	3.5 ± 0.9 (5)
b. phentolamine $5 \cdot 10^{-6}$ M present	2.4 ± 0.4 (8)	3.8 ± 0.9 (8)	4.0 ± 1.0 (8)	3.8 ± 1.0 (8)
c. propranolol $5 \cdot 10^{-6}$ present	1.9 ± 0.3 (10)	2.7 ± 0.6 (10)	3.2 ± 0.8 (8)	3.5 ± 0.7 (8)
4 Isoprenaline	1.7 ± 0.4 (6)	2.3 ± 0.8 (6)	3.5 ± 0.4 (6)	3.9 ± 0.3 (6)

d. Drugs

The drugs used were methoxamine HCl (Burroughs Wellcome & Co), noradrenaline bitartrate and adrenaline bitartrate (Sigma Chemical Co), isoprenaline (Minnesota 3M Lab.), phentolamine (Leopold Ciba) and propranolol (Federal 1CI).

Results

The spontaneous beating rate of the hearts in the perfusion apparatus ranged between 115 and 170/min. It usually declined somewhat during the course of the expts., as did the contractile force. The coronary flow ranged between 18 and 60 ml/min at the beginning of the expts. and was also found to be moderately decreased towards the end of the perfusion. The initial spontaneous outflow of PGE in the perfusate ranged between 0.8 and 5.1 ng/min. The outflow varied insignificantly between consecutive periods of effluent collection, provided the heart was allowed to beat spontaneously. In most expts. a weak tendency of the outflow of PGE to decline during the course of the expt. was observed. Thin layer chromatography of two lipid extracts revealed that its biological activity chromatographed with PGE₂. The outflow figures for PGE at rest and during infusion in the absence as well as in the presence of adrenolytic drugs, are presented in Table I. The chronotropic and inotropic responses to the amines are presented in Table II.

a. Methoxamine (MA)

Infusion of MA did not change the beating rate or the contractile force of the heart. The overflow of PGE during the infusion was reduced ($P < 0.05$) by $40 \pm 11\%$ ($n = 5$), but returned to the pre-infusion level after the drug infusion.

Discussion

A sympathetic nerve discharge and NA is liberated from the nerve endings, the synthesis and release of prostaglandins seems to be stimulated. This has been demonstrated as an increased overflow of PGE in the venous effluent from various tissues in response to sympathetic nerve stimulation (Davies *et al.* 1967, Gilmore *et al.* 1968, Shaw 1966, Wennmalm 1971). The mechanisms underlying this PGE release have not been clarified. It is not yet known whether the PGE is released from the nerve endings or from the vascular tissue. A direct release from the nerve endings into the synaptic cleft seems not probable since it has been shown that not only sympathetic nerve discharges but also infusion of NA (and A as well) elicits PG release into the effluent from sympathetically innervated organs (Gilmore 1968, Jansson and Wennmalm 1973). Consequently it appears likely that the synthesis and release of PGE during sympathetic nerve stimulation is one of the receptive functions triggered by the released neurotransmitter substance. It is not known for certain whether or not this receptive function involves the same part of cell surface in the synaptic cleft which is activated by the chemical stimulus and is conventionally named the adrenoceptor. However it has been reported, that pretreatment of the spleen with phentolamine, an adrenoceptor blocking agent, inhibits the release of PGE induced by the infusion of A (Davies *et al.* 1967). This might indicate that the PGE release should be bound to some of the adrenoceptor functions, possibly that commonly called to as the α -receptor. An alternative explanation would be that the mechanical force in the form of muscular activity directly or indirectly should facilitate the release of PGE. If so, the inhibited release of PGE by phentolamine in the dog spleen experiment mentioned above (Davies *et al.* 1967) should be secondary to the lack of contractile response to A. Supporting this latter alternative is the finding by Block and Vane (1971) that fibrillation in the isolated rabbit heart abolishes its basal release of prostaglandin substances. These authors concluded that the PGE release in the rabbit heart is correlated to the mechanical activity of the organ. On the other hand, hypertension or hypotension have been shown to cause a considerable inotropic response in rabbit hearts without affecting their basal release of PGE (Wennmalm 1975). These results do not support the idea of a causal relationship between PGE release and mechanical activity and prompt a more detailed analysis of the adrenoceptor role for the NA induced release of PGE in the heart.

The most efficient agent for the release of PGE tested in the current series was NA which infusion increased the overflow of PGE in the effluent to almost 4 times the basal value. After the infusion the PGE overflow returned to the resting level. Infusion of A also stimulated the synthesis and release of PGE, the overflow increasing to more than twice the basal value. Also after A infusion the increased PGE outflow returned to the initial level. In parallel to facilitating the PGE outflow infusion of NA and A elicited its well known stimulation of stroke frequency and contractile force, which persisted during the whole period of drug infusion. However the relationship between the overflow of PGE and the concomitant increase in the mechanical activity of the heart was not causal, for the following reason. After addition of phentolamine, an inhibitor of the α -response in the adrenoceptor

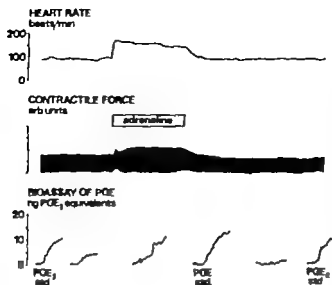


Fig. 1 Perfused rabbit heart. The top two tracings show heart rate and contractile force at rest and during infusion of adrenaline (maintained during the 10 min interval indicated by the box). The lower tracing demonstrates the contraction of the rat stomach strip when exposed to 1 μ g of PGE_2 (PGE_2 std), or to a smaller fraction of the dissolved extract of the effluent from the heart, collected during the 10 min period corresponding to the bioassay tracing.

The β -blocking agent propranolol completely counteracted the chronotropic response to A, but did not affect the inotropic response to the drug. No increase in the release of PGE_2 was obtained when A was infused in the presence of propranolol.

d Isoprenaline (IP)

IP elicited the most marked positive chronotropic response of the sympathomimetic amines investigated. The inotropic response to IP was moderate. IP did not affect the outflow of PGE_2 from the heart (Fig. 2).

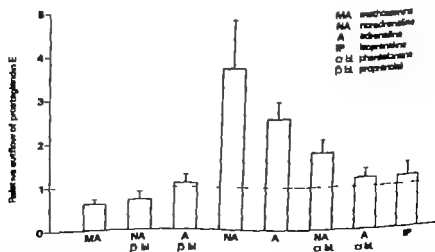


Fig. 2. Outflow of prostaglandin E from the isolated rabbit heart during infusion of sympathomimetic amines in the absence and in the presence of adrenergic agents. The PGE_2 outflow is expressed as the ratio between the outflow during drug infusion period and the mean outflow during the preceding and the following period. Relative outflow figures under the broken horizontal line consequently indicate that no increase in PGE_2 outflow was obtained during the drug infusion. The different sympathomimetic drugs are arranged from the left to the right in decreasing α -agonistic and increasing β -agonistic effect.

catecholamines, NA and A. Thus, in this respect the division of the adrenoceptor into α -types appears unuseful. The view of the adrenoceptor being a single physiological entity has been proposed by others (Ahlquist 1973).

The current results demonstrate that adrenaline and noradrenaline elicit PGE from the rabbit myocardium. This effect by the two drugs is clearly distinct from the positive chronotropic and inotropic effects caused by them. It is therefore concluded that the rabbit myocardium contains a hitherto unknown adrenoceptive mechanism stimulated by noradrenaline which on activation facilitates the bioformation of PGE in the tissue.

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to the perfusate prior to the infusions of NA or A, the increase in PGE outflow elicited by the amines was markedly reduced, although the chronotropic response to either drug and the inotropic response to A was unaffected. Furthermore, IP which on infusion elicited a pronounced increase in heart rate and contractile force, did not affect the overflow of PGE from the heart. Thus, the results do not support the hypothesis that the PGE outflow in the rabbit heart is due to the mechanical activity of the organ. On the other hand, it is demonstrated that the PGE releasing effect of NA and A is decreased by the pharmacologic agent which blocks the α response of the adrenoceptor which might support that at least part of the receptor function could be involved in the initiating mechanism for PGE release.

It thus appears that the rabbit myocardium contains receptors which can be activated by NA or A to stimulate the formation and release of PGE. Such receptors are by definition α -adrenoceptors. The current experiments permit a comparison between such α -adrenoceptor and the conventional adrenergic receptors for electro-mechanical or metabolic responses. The latter are denoted, according to the classification by Ahlquist (1948), as α_1 or α_2 , depending on their relative responsiveness to different sympathomimetic amines most closely related to A which at the time when this classification was suggested, was still believed to be the sympathetic neurotransmitter substance. Concerning the myocardial α -receptors, it was originally proposed by Ahlquist that they are of the β -type (Ahlquist 1948). Later, strong evidence has been presented supporting the existence of myocardial α -receptors (Govier 1968, Rabinowitz *et al.* 1975). The physiological significance of α -receptors in the heart has, however, been disputed (Nickerson 1973). In the present study the α -adrenolytic agent phentolamine completely inhibited the inotropic response to NA, and the α -adrenolytic agent propranolol completely inhibited the chronotropic but not inotropic responses to NA and A. These results support the hypothesis that the mechanical responses of the heart to catecholamines are not elicited via activation of myocardial β -adrenoceptors only. Since the scope of the current investigation was to study the adrenoceptive mechanism behind myocardial PGE synthesis stimulation these observations on the adrenergic mechanical responses of the heart, although *per se* interesting, were not studied more in detail.

MA, a drug considered to act only on α -adrenoceptors, was found inefficient to induce a chronotropic or inotropic response in the present study. The effect of MA infusion on the overflow of PGE in the myocardial effluent was slightly negative. This shows that a selective α -agonist is not the adequate stimulus for PGE release, in spite of the fact that this release was readily inhibited by the α -blocking drug phenoxybenzamine (Davies *et al.* 1967). The α -receptor blocking agent thus seemed to be able to inhibit other functions than the activation of α -receptors. A similar inhibition of the NA- or A-induced PGE release was found after pretreatment with propranolol, known as a potent inhibitor of the β -adrenergic response. The selective stimulation of β -receptor activity by IP did however not stimulate any PGE release. Obviously both kinds of pharmacological adrenoceptor inhibitors were demonstrated to block the PGE release although none of the artificial α - or β -agonist stimulant drugs were able to mimic the PGE releasing action of biological monoamines. In consequence the receptor for PGE release could not be identical with that conventionally named α - or β -receptor. Instead the results indicate that concerning the release of PGE the adrenoceptor constitutes a single functional unit that is

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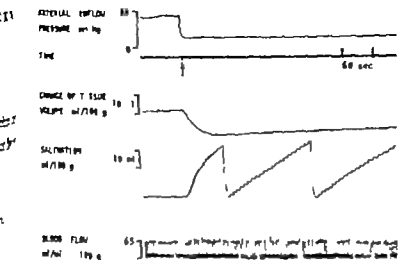


Fig. 1. Original tracings illustrating typical decrease of tissue volume occurring concomitant to the initial, rapid phase of salivation evoked by parasympathetic stimulation (20 Hz arrow) of the cat submandibular gland perfused at constant blood flow. perf. record by saline shows reset to control repeatedly.

During the initial 25–30 s and then gradually finished to be completed within 60 s, after this time, tissue volume instead showed a slight, continuous increase.

There was thus a close relation in time between the early rapid phase of salivation and the initial decrease of tissue volume in the depicted experiment. The cumulative decrease of tissue volume (Fig. 1) was 12.5 ml/100 g tissue. During the same period (~60 s) a total of 10 ml/100 g tissue of saliva was expelled. It seems reasonable to assume that the observed decrease of tissue volume reflects a corresponding output of saliva, an assumption supported by the finding that there was no significant change of tissue volume (regional blood content) in response to papaverine induced maximal dilatation of the constant flow perfused vascular bed. The remaining amount of saliva produced during the first minute of stimulation must thus have been derived from the blood and will then amount to 11.5 ml/100 g tissue (24 minus 12.5 ml/100 g). This figure corresponds almost exactly to the later steady state rate of salivation, which was 11.6 ml/min/100 g. A similar close correspondence was found also in the other stimulation experiments and it seems unlikely to be fortuitous but may indicate that the rate of fluid mobilization from plasma for subsequent saliva formation was approximately constant from the commencement of stimulation.

In summary it appears that the initial, rapid outflow of saliva in response to parasympathetic activation is partly due to mobilization of fluid from the gland as revealed in terms of a decline of tissue volume, and partly due to formation of saliva from fluid mobilized from the blood. Circumstantial evidence suggested that the latter process occurred at an approximately constant rate during stimulation. The decrease of tissue volume may to a significant extent reflect expulsion of preformed saliva from the salivary ducts caused by contraction of the myoepithelial cells (Emmelfin *et al.* 1974, 1976). It may also reflect osmotic attraction of cellular and/or interstitial fluid to the saliva (Schneyer 1975).

Dynamics of saliva secretion and tissue volume changes during parasympathetic stimulation in the constant flow perfused cat submandibular gland

By

JAN LUNDVALL and JAN HOLMBERG

The secretion evoked by parasympathetic stimulation of salivary glands is rapid initially and then slows down. In a recent study (Lundvall and Holmberg 1977) the initial rapid rate of salivation from the cat submandibular gland was found to coincide in time with a decrease of the tissue volume of the gland. The decline of tissue volume was present despite the regional blood volume in the gland must have increased during the evoked vasodilator response. In the present investigation an attempt was made to study in some detail the quantitative relation between tissue volume changes and rate of salivation in response to parasympathetic stimulation. The experiments were performed under conditions of constant blood flow perfusion in order to minimize alterations of the regional blood content in the gland.

The submandibular gland was dissected free unilaterally in 4 cats anesthetized with chloralose-urethane. The salivary duct was cannulated with a polyethylene catheter. Blood was shunted from the carotid artery via a Harvard peristaltic pump to the glandular artery. Venous outflow from the gland was diverted through an optical drop-recorder unit for continuous registration of blood flow and was then returned to the animal via a funnel connected to the right femoral vein. The distal end of the severed chorda-tongue nerve was fitted in a bipolar ring electrode. The gland was placed in a hermetically sealed, temperature-controlled (38°C) plethysmograph filled with Tyrode solution. Arterial inflow pressure was measured from a T-tube in the arterial shunt. The nerve was stimulated with square wave pulses of 1 ms duration at a rate of 20 Hz. The drops of saliva fell into a cup, the weight of which was continuously registered with a transducer. Tissue weight changes were transformed into volume changes by calibration. Papaverine was infused close arterial in a dose causing maximal vasodilatation in order to study the effect on tissue volume of vasodilatation such. Arterial blood pressure, changes of tissue volume, rate of salivation and blood flow were registered on a Grass Polygraph.

Fig. 1 shows an original record from a typical experiment. Blood flow was held constant at 65 ml/min. 100 g glandular tissue. Control blood pressure was 135 mmHg and it fell abruptly upon chorda activation (20 Hz, arrow) to a maintained low level of about 43 mmHg. It can be seen that tissue volume started to decrease almost immediately upon commencement of stimulation and that, with a few seconds delay, salivation was induced. The salivary production was rapid initially and then gradually declined to attain a maintained, approximately steady-state level after about 60 s. Similar results were obtained in 10 other experiments.

Polyamines and nucleic acids in the mouse kidney Induced to growth by testosterone propionate

By

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Abstract

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Only injection of testosterone propionate to castrated mice resulted in a striking increase in kidney weight, and putrescine rose sharply and the amounts of spermidine and spermine are also increased. The activity of ornithine decarboxylase is enhanced to values of more than 1 000 times the control level after few days of testosterone substitution. A moderate and temporary increase in the activity of the putrescine-derived S-adenosyl-L-methionine decarboxylase is observed. Testosterone injections produced large amounts of renal RNA but only minor changes in DNA. It is apparent that in mice distinct alterations in polyamine metabolism occur during the development of renal hypertrophy induced by testosterone administration.

Key words: Polyamines, nucleic acids, testosterone propionate, renal hypertrophy

A distinct difference in the rate of formation and excretion of putrescine between male and female mice has been disclosed (Henningsson and Rosengren 1975, 1977). This difference was accounted for by a high activity of ornithine decarboxylase, the enzyme catalyzing the formation of putrescine, in the male kidney. Castration produced a rapid decrease in ornithine decarboxylase activity whereas on the other hand substitution with testosterone resulted in an increased formation of putrescine.

The significance of changes in the levels of putrescine and the polyamines, spermidine and spermine, and the activities of their synthesizing enzymes has recently been much discussed. The biosynthesis and the accumulation of putrescine and polyamines undergo striking changes in processes related to rapid cell growth, e.g. in liver at regeneration after partial hepatectomy or growth hormone administration, in developing chick embryo, in renal and myocardial hypertrophy and in malignant growth (for ref. see Raina and Järnefelt 1973, Tabor and Tabor 1976). These and related observations have called attention to the possibility of polyamines being implicated in RNA and DNA metabolism. However the physiological role of these amines in mammalian tissues is unknown.

Like workers before us, we observed that in mice castration resulted in a considerable

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Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate

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Abstract

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My sections of testosterone propionate in castrated mice resulted in striking increase in kidney weight. Putrescine rose sharply and the contents of spermidine and spermine were also increased. The activity of ornithine decarboxylase was enhanced to values of more than 1 000 units the control level within few days of testosterone substitution. A moderate and temporary increase in the activity of the putrescine-specific 3-adenosyl-L-methionine decarboxylase as observed. Testosterone injections produced large amounts of renal RNA but only minor change in DNA. It appears that in mice distinct alterations in polyamine metabolism occur during the development of renal hypertrophy induced by testosterone administration.

Key words: Polyamines, nucleic acids, testosterone propionate, renal hypertrophy

A distinct difference in the rate of formation and excretion of putrescine between male and female mice has been disclosed (Henningsson and Rosengren 1975, 1977). This difference was accounted for by a high activity of ornithine decarboxylase, the enzyme catalyzing the formation of putrescine, in the male kidney. Castration produced a rapid decrease in ornithine decarboxylase activity whereas on the other hand substitution with testosterone resulted in an increased formation of putrescine.

The significance of changes in the levels of putrescine and the polyamines, spermidine and spermine, and the activities of their synthesizing enzymes has recently been much discussed. The biosynthesis and the accumulation of putrescine and polyamines undergo striking changes in processes related to rapid cell growth, e.g. in liver at regeneration after partial hepatectomy or growth hormone administration, in developing chick embryo, in renal and myocardial hypertrophy and in malignant growth (for ref. see Raine and Jänne 1975, Tabor and Tabor 1976). These and related observations have called attention to the possibility of polyamines being implicated in RNA and DNA metabolism. However the physiological role of these amines in mammalian tissues is unknown.

Like workers before us, we observed that in mice castration resulted in a considerable

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- SCHNEIDER, L. H. Secretion of K and fluid by rat submaxillary during sympathetic nerve stimulation. *J Physiol* 1975 229 1056-1061.

— *isolation of polyamine spermidine and spermine in kidney extracts*

— *isotopic separation and quantitative estimation of the isolates in kidney extracts were carried by high resolution liquid chromatography using the automatic amino acid analyzer LKB-BIOCAL. The method as described by Hemington (1977), was used with minor modifications.*

— *isolation and determination of nucleic acids and proteins*

— *isolation of RNA and DNA was performed by the Schmidt and Thannhauser (1945) procedure as modified by Schneider (1945). The kidney tissue was homogenized in 7 volumes of ice cold water. An aliquot of the homogenate (0.8 ml) was precipitated with 2.5 ml of ice cold 10% (w/v) trichloroacetic acid. The mixture was centrifuged at 900 g for 15 min at 0°C. The supernatant was removed and the pellet was washed three times with 2.5 ml of 10% trichloroacetic acid. Thereafter the pellet was suspended in 5 ml 1 M KOH and the mixture was hydrolyzed for 18 h at 37°C. The hydrolysate was neutralized and the mixture was reprecipitated in another 5 ml of trichloroacetic acid and centrifuged. The combined supernatants contained the ribonucleic acid (RNA) fraction while DNA and protein were trapped on the pellet.*

— *The amount of RNA was estimated using the orcinol method (Majumdar 1959). Briefly this was achieved by mixing the RNA extract with orcinol after which the mixture was boiled in water-bath for 20 min. Optical density was measured in a Zeiss spectrophotometer.*

— *For DNA the pellet was suspended in 5 ml of 5% trichloroacetic acid and the mixture was heated to 50°C for 15 min. After centrifugation, the pellet was resuspended once in trichloroacetic acid. The resulting supernatants which formed the DNA fraction were mixed with 5.2 M HClO₄ and diphenylpicrylhydrazyl reagent (Richards 1974). The resulting solution was incubated at 30°C for 18 h and DNA was estimated colorimetrically (Barton 1954).*

— *Protein was determined by the Folin procedure (Lowry et al. 1951). — The standards used were RNA and DNA from calf thymus and for protein determination bovine serum albumin. These standards were purchased from Sigma Chemical Co.*

Results

— *Changes in weight and protein mass of the kidney in mice during testosterone administration*

— *In kidney of the male mouse lost about 30% in weight within 3 weeks of castration, i.e. weight loss from 387 ± 8.1 mg in 16 normal mice to 275 ± 5.0 mg in 32 castrated ones. Reversal of castrated mice to testosterone propionate resulted in a marked increase of the kidney weight (Fig. 1). The weight was significantly elevated already 12 h after the first injection. Daily injections of testosterone produced a successive increase in weight during the whole period of observations, this increase was at first steep then more slow. Fig. 1 also shows that simultaneously with the increase of kidney weight a parallel increase in the content of renal protein occurred indicating that the weight increase was due to real growth of the kidney. The total amount of renal protein was reduced from 77.4 ± 2.19 mg in normal mice to 53.9 ± 2.66 mg in castrated ones. It should be mentioned that a small and possibly not statistically significant.*

— *The effect of testosterone on the levels of RNA and DNA in the kidney*

— *The changes in RNA and DNA contents in the kidney of castrated mice after substitution with testosterone propionate are shown in Fig. 2. After testosterone administration the content of RNA rose steeply during the first 3 days and continued to increase throughout*

loss in kidney weight a change which could be reversed by testosterone substitution. Selby (1939) was first to describe a renal hypertrophy during testosterone injections. Under androgen stimulation, the kidney cells become larger without apparent mitotic activity (Kleber *et al.* 1940). Kochakian and Harrison (1962) showed that testosterone injections in castrated mice produced a large increase in renal RNA but only minor changes in DNA. Hence, the mouse kidney appears to be an organ of choice in studies on the role of di- and polynucleotides as related to the metabolism of RNA and protein as discerned from the metabolism of DNA.

Methods

Male mice of the NAFI strain were used. They were fed a standard pellet diet and had water *ad libitum*. Gonadectomy was performed at the age of 8 weeks. Three weeks later testosterone administration began in the form of daily subcutaneous injections of 200 μ g testosterone propionate (British Drug Houses Ltd., Poole, England) suspended in 50 μ l of rachis oil. Castrated controls were given rachis oil only. Mice subjected to testosterone injections as well as controls were sacrificed at varying times throughout the experiments.

Preparation of samples for analysis

Mice were stunned and exsanguinated. The removed kidneys, from which the capsules are gently peeled off, were blotted on gauze, weighed and promptly placed in a beaker cooled on ice.

Extracts for assay of ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities were prepared in the following way. The kidneys were minced with scissors and part of the tissue was immediately and cautiously homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 7 volumes of cold sodium phosphate buffer (0.1 M, pH 7.2) containing EDTA (final conc. 10^{-4} M), dithiothreitol (final conc. $5 \cdot 10^{-4}$ M) and glucose (0.2% w/v). The homogenate was centrifuged at 20 000 g for 20 min at 4°C. The supernatant was decanted and used.

Extracts for determination of putrescine, spermidine and spermine in the kidney tissues are prepared as follows. The minced tissues were homogenized in 9 volumes of a solution of 4% sulfosalicylic acid and 0.04% EDTA. The extract was boiled on a water-bath for 30 min whereupon the mixture is chilled and centrifuged. The pH of the sample was adjusted to 0–2.5 with NaOH and the extract was filtered through a filter with a pore size of 0.22 μ m (Millipore Corp., Bedford, Mass. 01730).

Determination of ornithine decarboxylase activity

Ornithine decarboxylase activity was determined measuring the release of $^{14}\text{CO}_2$ from carboxyl-labelled ornithine. An aliquot of the enzyme extract corresponding to 5 mg of tissue was incubated in a reaction mixture containing ^{14}C -carboxyl-labelled DL-ornithine monohydrochloride (final conc. 10^{-4} M, sp. act. 0.5 mCi/mmol), pyridoxal 5-phosphate (final conc. 10^{-6} M) and the same phosphate buffer as used for homogenization. The total volume of the incubate was 1.0 ml. The mixture was incubated for 30 min at 37°C. The reaction was terminated by tipping 0.5 ml of 2 M perchloric acid from a side arm of the incubation vessel. Blanks were treated in the same way except that tissue was excluded. The expelled $^{14}\text{CO}_2$ is trapped on a 10 \times 25 mm piece of No. 005 Munktell filter paper prepared with 100 μ l of hydrazine of Hyamine 10-X (1 M solution in methanol). Maximal absorption of $^{14}\text{CO}_2$ was achieved by continuing shaking for another 45 min. The filter paper was then placed in a counting vessel containing 3 ml of Bray liquid scintillation mixture (Bray 1960) and its radioactivity was measured in a Packard Tri-Carb Liquid Scintillation spectrometer.

Determination of putrescine-activated S-adenosyl-L-methionine decarboxylase activity

Enzyme activity was determined by measuring the release of $^{14}\text{CO}_2$ from carboxyl-labelled S-adenosyl-methionine in the presence of putrescine according to the method described by Fegg and Williams-Ashman (1969). The incubation mixture contained enzyme extract corresponding to 75 mg of tissue, putrescine (final conc. $2.5 \cdot 10^{-4}$ M), ^{14}C -carboxyl-labelled S-adenosyl-L-methionine (final conc. 10^{-4} M, sp. act. 0.5 mCi/mmol) and the same buffer as used for homogenization. The total volume of the incubation mixture was 1.0 ml and the mixture was incubated for 1 h at 37°C. Blanks were obtained by incubating the same reaction mixture without tissue. The $^{14}\text{CO}_2$ evolved was trapped and counted as described above for ornithine decarboxylase.

TABLE I. Effect of testosterone administration on the concentrations of RNA, DNA, putrescine, spermidine and spermine in the kidney of castrated mice. The concentrations of RNA and DNA are given $\mu\text{g/g}$ and the concentrations of the amines in nmol/g . Means and the S.E. of mean are given. Number of observations in parentheses. $a^* p < 0.05$ $b^* p < 0.01$ $c^* p < 0.001$ versus controls.

Group of animals	RNA	DNA	Putrescine	Spermidine	Spermine
Normal mice					
1	5.8 ± 0.09 (16)	5.8 ± 0.14 (16)	21 ± 2.1 (12)	380 ± 18.0 (12)	855 ± 30.8 (12)
2	6.4 ± 0.28 (8) ^a	5.5 ± 0.23 (8)	90 ± 7.7 (12) ^a	411 ± 22.0 (12)	815 ± 8.7 (12)
3	6.4 ± 0.12 (8) ^a	5.5 ± 0.29 (8)	112 ± 6.3 (12) ^a	377 ± 19.3 (12)	815 ± 26.7 (12)
4	6.9 ± 0.19 (8) ^a	5.8 ± 0.18 (8) ^a	151 ± 7.0 (12) ^a	401 ± 16.6 (12)	868 ± 17.0 (12)
5	7.0 ± 0.17 (8) ^a	5.8 ± 0.11 (8) ^a	159 ± 12.8 (12) ^a	439 ± 29.4 (12) ^a	834 ± 20.4 (12)
6	7.2 ± 0.24 (8) ^a	4.7 ± 0.22 (8) ^a	154 ± 9.9 (12) ^a	460 ± 18.3 (12) ^a	903 ± 20.2 (12)
7	7.5 ± 0.13 (8) ^a	4.4 ± 0.06 (8) ^a	139 ± 7.1 (12) ^a	449 ± 17.7 (12) ^a	1005 ± 41.0 (12) ^a
8	6.9 ± 0.13 (8) ^a	3.9 ± 0.10 (8) ^a	163 ± 15.4 (12) ^a	447 ± 15.2 (12) ^a	935 ± 11.0 (12) ^a
9	7.1 ± 0.09 (8) ^a	3.8 ± 0.10 (8) ^a	172 ± 6.4 (12) ^a	399 ± 10.7 (12)	896 ± 17.5 (12)
Castrated mice					
10	6.2 ± 0.24 (5)	4.8 ± 0.15 (5) ^b	49 ± 8.3 (6) ^c	377 ± 28.1 (6)	874 ± 30.0 (6)

of DNA concentration. These changes in the levels of nucleic acids testify to hypertrophy as the major cellular process of the renal enlargement. — After castration the total amount of renal DNA was reduced (Fig. 2 and Table I). However this reduction was proportionally less than the weight loss of the kidney. Consequently the concentration of DNA was increased in castrated mice in comparison with normal mice. The total amount of RNA was reduced after castration while no significant change in RNA concentration was observed.

Ornithine decarboxylase and 5-adenosyl-L-methionine decarboxylase activities in castrated mice after testosterone substitution

The rate of putrescine formation, *i.e.* the ornithine decarboxylase activity in the kidney of normal male mice was 3.5 ± 0.52 nmol/mg protein \cdot h (5 determinations). 3 weeks after castration, putrescine formation had fallen and was too low to be detectable (10 determinations). To examine the time course of changes in renal ornithine decarboxylase activity after injections of testosterone propionate, groups of 5 mice were killed at different intervals between 3 h and 21 days after the first injection. Results are summarized in Fig. 3. An elevation in enzyme activity was noted 6 h after testosterone substitution the observed figure was 0.2 ± 0.10 nmol/mg protein \cdot h (5 determinations). During the first 3 days the activity was well above 200 nmol/mg protein \cdot h. Further increase in ornithine decarboxylase activity during the remainder of the experimental period was slight.

5-Adenosylmethionine decarboxylase activity in extracts of kidney from castrated mice was significantly higher than in extracts of kidney from normal mice (Fig. 4). Substitution with testosterone propionate after castration resulted in a transient increase of enzyme activity reaching a maximum of about 30% between days 1 and 3 of treatment. After 7 days the enzyme activity had declined to values below the controls however the difference was not statistically significant.

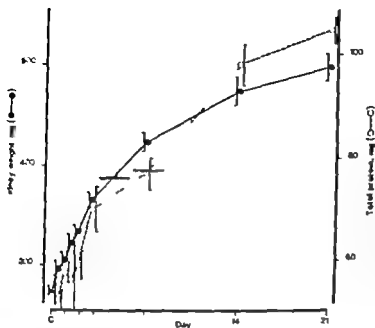


Fig. 1. Weight and total protein of the kidney of castrated mice after daily injections of 200 μ g testosterone propionate. Each point is the mean \pm S.E. of at least eight observations or more; each point for total protein is the mean \pm S.E. of at least eight observations or more. The vertical lines define the S.E. of the mean; the horizontal bars indicate the deviation for the normal male rat; C stands for castrated controls.

the whole period of observations. In contrast with the rapid and sharp elevation of RNA content, no alteration of DNA content was observed during the first 3 days of treatment. Later *i.e.* between 1 and 3 weeks of injections, a moderate enhancement of DNA content appeared.

The concentration of renal RNA gradually increased during the first week of testosterone injections and then decreased (Table I). Administration of testosterone gave rise to a red

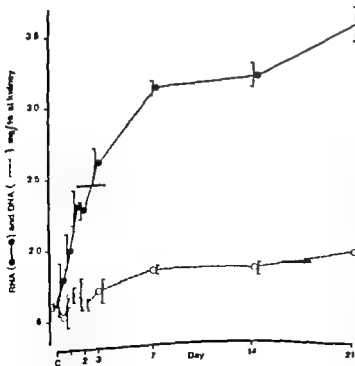


Fig. 2. Total amounts of RNA and DNA in the kidney of castrated mice after daily injections of 200 μ g testosterone propionate. Each point is the mean \pm S.E. of at least eight observations. The horizontal bars indicate the total amount of RNA and DNA in the kidney of normal mice (5 observations). C stands for castrated controls. RNA was significantly increased at 24 h ($p < 0.001$) and DNA on the 7th day ($p < 0.05$).

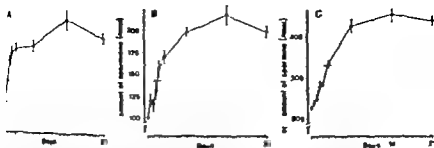


Fig. 1. Total amounts of putrescine (A), spermidine (B) and spermine (C) in the kidney of castrated mice 12 days after injection of 200 μ g testosterone propionate. Each point is the mean and S.E. of the mean of 10 mice. The horizontal bars indicate values for normal male mice (6 observations). C stands for control.

jected with testosterone propionate. After 48 h the total amount of putrescine in the kidney was 10 times greater and the concentration was 8 times greater than in the control. During the following 3 weeks of observation no further alteration in the concentration of putrescine occurred. However as the kidney weight steadily increased, the total amount of putrescine was increased. Furthermore, administration of testosterone led to a marked increase in the total amounts of spermidine within 12 h and of spermine within 72 h. Within 72 h the total amount of spermidine increased by 70% and that of spermine by 50%. A further moderate rise of the contents of both amines occurred during the following period of observation. As to the concentrations of spermidine and spermine there was a slight elevation with the highest values on day 7.

Discussion

The increase in kidney weight of castrated mice after testosterone substitution was found to be associated with an increase in the content of protein in the kidney in good agreement with the findings of Kochakian and Stettin (1948) indicating growth and not edema as the origin of enlargement. Selby (1939) and Pfeiffer *et al.* (1940) attribute the enlargement of the kidney after androgen treatment to a hypertrophy of the cells without any apparent mitotic activity. This is in accordance with the accumulation of RNA and the linked change in protein content after testosterone administration. These findings agree with the results of Pfeiffer *et al.* (1942) and Kochakian and Harrison (1962). A dose-response curve (to be published) for testosterone propionate in the range of 100 to 400 μ g/mouse/day showed a progressive increase in kidney weight and ornithine decarboxylase activity with increasing doses of testosterone. Up to a daily dose of 200 μ g testosterone propionate the putrescine formation and kidney weight rose rapidly. With higher daily doses the increase in putrescine formation and kidney weight was small. Consequently the dose of 200 μ g/mouse/day was chosen in the present study. The administration of the kidneys by testosterone resulted in a marked increase of ornithine decarboxylase activity. Decarboxylation of ornithine is considered to be the rate-limiting step in the biosynthesis of polyamines (Jänne and Raina 1968, Williams-Ashman *et al.* 1969).

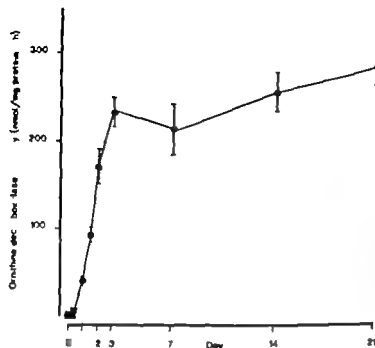


Fig. 3 Ornithine decarboxylase activity (nmol/mg protein/h) in the kidney of castrated mice after daily injections of 200 μ g testosterone propionate. Each point represents the mean and S.E. of the mean of 10 observations except for the control group (C) which is the mean of 10 observations.

Contents of putrescine, spermidine and spermine in the kidney of castrated mice after testosterone administration

The total amounts of putrescine, spermidine and spermine as well as the putrescine concentration in the kidney of castrated mice were considerably lower than in the kidney of normal mice, while concentrations of spermidine and spermine remained unchanged (Table I).

An early and marked accumulation of renal putrescine occurred when castrated

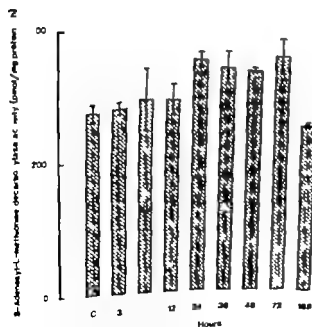


Fig. 4 S-Adenosyl-L-methionine decarboxylase activity (nmol/mg protein/h) in the kidney of castrated mice at different times after the first injection of 200 μ g testosterone propionate. Each column represents the mean and S.E. of the mean of at least 5 observations. C for castrated controls and normal male mice. * $p < 0.01$ versus controls.

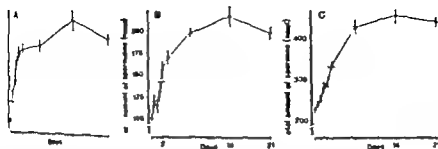


Fig. 3. Total amounts of putrescine (A), spermidine (B) and spermine (C) in the kidney of castrated mice at daily injections of 200 μ g testosterone propionate. Each point is the mean and S.E. of the mean of 10 animals. The horizontal bars indicate values for normal male mice (6 observations). C stands for control.

or injected \blacksquare testosterone propionate. After 48 h the total amount of putrescine in the kidney was 10 times greater and the concentration was 8 times greater than in the control kidney. During the following 3 weeks of observation no further alteration in the concentration of putrescine occurred. However as the kidney weight steadily increased, the total amount of putrescine was increased. Furthermore, administration of testosterone led to a significant increase in the total amounts of spermidine within 12 h and of spermine within 24 h. Within 72 h the total amount of spermidine increased by 70% and that of spermine by about 40%. A further moderate rise of the contents of both amines occurred during the following period of observation. As to the concentrations of spermidine and spermine there was a slight elevation with the highest values on day 7.

Discussion

The increase in kidney weight of castrated mice after testosterone substitution was found to parallel an increase in the content of protein in the kidney in good agreement with the results of Kochakian and Satterer (1948) indicating growth and not edema as the origin of renal enlargement. Schyc (1939) and Pfeiffer *et al.* (1940) attribute the enlargement of the kidney after androgen treatment to a hypertrophy of the cells without any apparent mitotic activity. This is in accordance with the accumulation of RNA and the limited change in DNA content after testosterone administration. These findings agree with the results of Kamekura *et al.* (1962) and Kochakian and Harrison (1962).

A dose-response curve (to be published) for testosterone propionate in the range of 0.1–1000 μ g/mouse day showed a progressive increase in kidney weight and ornithine decarboxylase activity with increasing doses of testosterone. Up to a daily dose of 200 μ g of testosterone propionate the putrescine formation and kidney weight rose rapidly. With larger daily doses the increase in putrescine formation and kidney weight was small. Consequently the dose of 200 μ g/mouse day was chosen in the present study.

Stimulation of the kidneys by testosterone resulted in a marked increase of ornithine decarboxylase activity. Decarboxylation of ornithine is considered to be the rate-limiting step in the biosynthesis of polyamines (Jilka and Rahm 1968, Williams-Ashman *et al.* 1969).

It is noteworthy that an increase in ornithine decarboxylase activity was detected as early as 6 h after the injection of testosterone. Further, the greatest enhancement of this enzyme occurred during the first two days of observation while the changes of the other parameter studied occurred less abruptly. This is interesting because a connection between polyamine and growth is assumed to exist (for ref. see Russell 1973, Raina and Jänne 1975).

The enhancement of the activity of ornithine decarboxylase was impressive: within a few days of testosterone treatment the activity rose to values of more than 1 000 times the control level. The ornithine decarboxylase activity of the testosterone-stimulated mouse kidney appears to be singularly high, as it was found about 5 times larger than the highest value recorded in rat tissues, i.e. the ovary stimulated by chorionic gonadotropin (Coté and Jänne 1977).

The testosterone-induced increase of ornithine decarboxylase activity was accompanied by a rise in putrescine content. However, the increase of putrescine content was out of proportion to the great enhancement of enzyme activity. This could be due to an increased turnover of the amine. Urinary excretion of putrescine was found elevated after testosterone administration (Grahn *et al.* 1973). Another possibility is that the supply of ornithine is inadequate to the enormous activity of ornithine decarboxylase in testosterone-treated mice. Interestingly, Kochakian (1945) reported an increase in arginase activity (catalyzing the synthesis of ornithine and urea) in androgen-stimulated mouse kidney. This would result in more ornithine available for decarboxylation and hence for biosynthesis of polyamines.

The change in the activity of the second enzyme in polyamine biosynthesis, S-adenosylmethionine decarboxylase, after testosterone administration was comparatively small. However, the elevated concentration of putrescine, which *in vitro* is known to stimulate the activity of S-adenosylmethionine decarboxylase (Pegg and Williams-Ashman 1968), should give rise to an increased enzyme activity *in vivo* and hence, an elevated synthesis of polyamines. It should be noticed that S-adenosylmethionine decarboxylase activity was significantly ($p < 0.05$) higher in the kidneys of castrated mice than in normal mice.

The changes in spermidine and spermine concentrations after treatment with testosterone appeared to parallel the alteration in RNA concentration. This is interesting with regard to the numerous reports indicating a relationship between polyamines and nucleic acids.

The rat ventral prostate gland is another organ showing responsiveness to castration and androgen treatment (Pegg *et al.* 1970). However, the changes in polyamine metabolism, after gonadectomy or androgen treatment, differ considerably from those in the kidney. Among the most outstanding ones are the contrasts in enzyme activities. The ornithine decarboxylase activity was increased only a few times after testosterone administration, whereas S-adenosylmethionine decarboxylase activity was enhanced tenfold. The differences are difficult to explain, but it should be kept in mind that the growth of the ventral prostate after androgen treatment is a combination of hypertrophy and hyperplasia, distinct from the kidney.

Due to hypertrophy after testosterone administration, the mouse kidney appears to be a valuable organ in studying the importance of polyamines in RNA metabolism.

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Time course of light induced changes in pilocarpine sensitivity of rat iris

By

HELEN CLAEISSON and ERNST BÁRÁNY

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Abstract

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Pigmented rats were kept under different light regimens for 1-20 days. Supramaximal light was used to elicit a response of the iris sphincter in enucleated eyes with their cornea intact was tested *in vitro*. Pilocarpine $6 \cdot 10^{-5}$ M was used as standard stimulus. In agreement with previous authors there was a marked difference between constant light treated (subsensitive) eyes and dark treated (supersensitive) ones. As pretreated to reach maximal sub- and supersensitivity and then shifted to the opposite regimen, it was found that reversal was a gradual process starting within a few hours. Full reversal had not been reached after 72 h. The two curves of sensitivity vs time intersected around 4 h. The time constants of the two curves were approximately equal. Light regimens used: 1) intermittent light shifting every 1, 3, 6 or 12 h gave intermediate results.

Key words: Pilocarpine, iris, subsensitivity, light, time course.

Bitó and Dawson (1970) and Bitó *et al.* (1971) have shown that the sensitivity of the iris sphincter to cholinergic stimulation varies—whether tested *in vitro* or *in vivo*—as a function of environmental light intensity.

We have studied the time course for this modulation of sensitivity using the method of Beaver and Riker (1962) also used by Bitó.

Our main findings are that 1) changes reach a steady state only after about a week and 2) the time course for rising and falling sensitivity are not markedly different.

Materials and Methods

Pigmented Lister Hooded male rats were from OLAC, Shaw Farm, Oxon, England and arrived at the laboratory at the age of 4 weeks. At time of arrival the weight of the rats was about 180 g. They were put into the animal house which had alternating light and darkness, shifting every 12 h, where they were allowed to adapt for at least one week. The time the rats spent in the animal house, before entering an experiment was from one week to three weeks, which meant their weights varied from about 250 g to 325 g. This was found to influence the results.

In vitro test of sensitivity

After treatment the rats, three at a time, were picked up at random out of their treatment group. A 0.5 ml intraperitoneal injection of pentobarbital (veterinary medicinal) 60 mg/kg (A. S. Ernt) and decapitated.

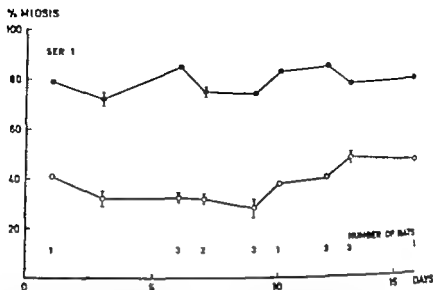


Fig. 2. Ser. 1 The effects of continuous darkness (●) and light (○) on the sensitivity (miotic response) of rat eyes. The points represent the main response in the number of rats shown in the lower part of the figure. Vertical bars indicate \pm S.E. If this is less than 1% it is hidden in the circles.

Series 3 Two groups of rats, 33 and 28 respectively were pretreated with continuous light and dark for 9 days (216 h) before being shifted to the opposite regimen. At time zero in their new regimen were sacrificed and the rest of them after 3–7 h of the new treatment.

Series 4 This time 70 rats were put into constant light only and killed after 9–13 days of treatment.

Results

The results from *series 1* showed a distinct difference in sensitivity of the iris sphincter between constant light and dark treated animals (Fig. 2). This difference in miosis was already after only 24 h of treatment, and did not change appreciably as time for treatment was prolonged.

In *series 2* the light treated rats showed a decrease in sensitivity as number of treatment days increased above 6, in the dark treated ones, the miosis was almost constant during experiment, possibly because it was so near maximum (Fig. 3). Alternating light and dark produced a sensitivity that was almost independent of treatment duration above 6 days as well as of the various cycle lengths and all animals showed a degree of miosis intermediate between that of light and dark treated ones.

Series 3 demonstrated that it took more than 72 h for full reversal of the light or dark induced changes. Reversal was a gradual process and the two curves intersected at about day 7 (Fig. 4).

Series 4 was run as a complement of *series 1* and *2*. All rats, independent of treatment time, 9–13 days showed approximately the same miotic response, a response which was within the range of the previous results from *series 1* and *2*.

A few experiments have further been run to examine the effect of even longer treatment. 3 days of treatment sensitized the animals just as much as 10–15 days (rats).

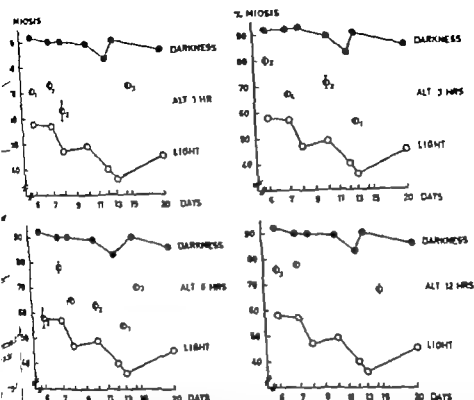


Fig. 3. Series 2 compares the effects of constant light (O), darkness (●), and alternating light and darkness (○). Each circle represents the mean response in the number of rats shown to the right of the points representing alternating light and darkness. For darkness treated ones (cf Fig. 4) the points represent 2, 1, 1, 2, 1 rats and for light treated 2, 4, 2, 2, 3, 1 rats. Vertical bars indicate ± S.E. (as in Fig. 2). Dotted lines represent the myosis effects of the other alternating treatments shown in the other panels. The points representing continuous light and darkness are shown with their S.E. in Fig. 4.

Discussion

The pilocarpine concentration $6 \cdot 10^{-4}$ mol/l was chosen so as to make the sub- and the supersensitivity responses lie symmetrically on the dose-response curve as in series 1. For unknown reasons, the degrees of myosis in the later series were higher and the range of variation no longer centered around 50% myosis. This introduces a bias into the comparison between the time courses of recovery from darkness and light (series 3): one underestimates the rate of change in the initial parts of the recovery from dark treatment. Despite this difficulty there is a striking similarity in the temporal development of supersensitivity and in that of subsensitivity. The changes progress slowly and steady state seems to be reached only after about a week. But it is not certain that the degree of sub- or supersensitivity reached is determined by the effector organ proper, the smooth muscle of the sphincter. Excitation of the sphincter in our experiments is by way of the pupillary light reflex and it could well be that other stations in the reflex arc also suffer changes during continuous light or darkness, subsensitivity during continuous activity and maybe even spontaneous activity after

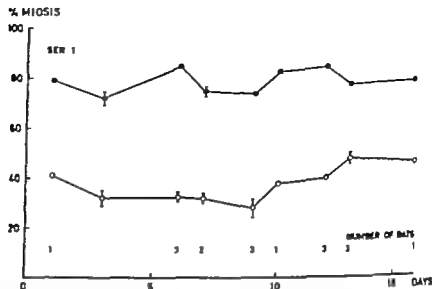


Fig. 2. Ser. 1 The effects of continuous darkness (●) and light (○) on the sensitivity (mitotic response) rat eyes. The points represent the main response in the number of rats shown in the lower part of the fig. Vertical bars indicate \pm S.E. If this is less than 1% it is hidden in the circles.

Series 3 Two groups of rats, 33 and 28 respectively were pretreated with continuous light and dark for 9 days (216 h) before being shifted to the opposite regimen. At time zero in their new regimen 8 were sacrificed and the rest of them after 3–72 h of the new treatment.

Series 4 This time 20 rats were put into constant light only and killed after 9–13 days of treatment.

Results

The results from *series 1* showed a distinct difference in sensitivity of the iris sphincter between constant light and dark treated animals (Fig. 2). This difference in mitosis was visible already after only 24 h of treatment, and did not change appreciably as time for treatment was prolonged.

In *series 2* the light treated rats showed a decrease in sensitivity as number of treatment days increased above 6. In the dark treated ones, the mitosis was almost constant during expt., possibly because it was so near maximum (Fig. 3). Alternating light and dark produced a sensitivity that was almost independent of treatment duration above 6 days well as of the various cycle lengths and all animals showed a degree of mitosis intermediate between that of light and dark treated ones.

Series 3 demonstrated that it took more than 72 h for full reversal of the light or darkness induced changes. Reversal was a gradual process and the two curves intersected at about 24 h (Fig. 4).

Series 4 was run as a complement of series 1 and 2. All rats, independent of treatment time, 9–13 days showed approximately the same mitotic response, a response which was within the range of the previous results from series 1 and 2.

A few expts. have further been run to examine the effect of even longer treatment. 30–40 days of treatment sensitized the animals just as much as 10–15 days (6 rats).

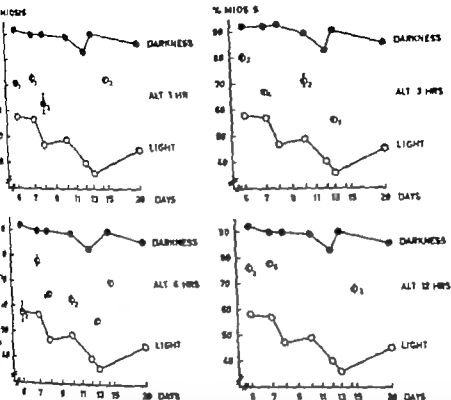


Fig. 1. Sec. 2 compares the effects of constant light (○), darkness (●), and alternating light and darkness (○). Each curve represents the mean response to the number of miosis shown to the right of the axis representing alternating light and darkness. For darkness treated eyes (cf. Fig. 4) the points represent 1, 2, 1, 1, 2, 1 rats and for light treated 2, 4, 2, 2, 3, 1 rats. Vertical bars indicate \pm S.E. (as in Fig. 2). Dotted lines represent the smooth effect of the other alternating treatments shown in the other panels. The points representing continuous light and darkness are shown with their S.E. in Fig. 4.

Discussion

The pilocarpine concentration $6 \cdot 10^{-4}$ mol/l was chosen so as to make the sub- and the supersensitivity responses lie symmetrically on the dose-response curve as in series 1. For unknown reasons, the degrees of miosis in the later series were higher and the range of variation no longer centered around 50% miosis. This introduces a bias into the comparison between the time courses of recovery from darkness and light (series 3): one underestimates the rate of change in the initial parts of the recovery from dark treatment. Despite this difficulty there is a striking similarity in the temporal development of supersensitivity and in that of subsensitivity. The changes progress slowly and steady state seems to be reached only after about a week. But it is not certain that the degree of sub- or supersensitivity reached is determined by the effector organ proper, the smooth muscle of the sphincter. Excitation of the sphincter in our experiments is by way of the pupillary light reflex and it could well be that other stations in the reflex arc also suffer changes during continuous light or darkness, subsensitivity during continuous activity and maybe even spontaneous activity after

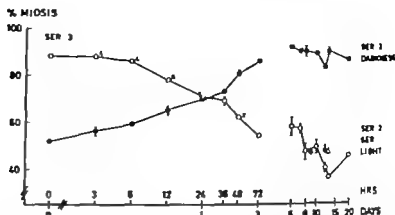


Fig. 4 Ser 2 is shown here to make it possible to compare ser 2, 3 and 4, see further the legend of Fig. 3. Ser 3 shows the responses in rats pretreated with 9 days of constant light and darkness and then shifted to the opposite regimen. The same symbols for different treatments are used as in Fig. 2 and 3, also vertical bars indicate \pm S.E. and the number of rats is indicated to the right of the dots. Note the intersection between the curves at about 4 h. Ser 4 is rats that have been light treated for 9, 12 and 13 (Δ). The triangles represent 9, 6 and 5 rats.

prolonged total darkness. The changes observed *in vitro* thus are the minimum that constant excitation or constant lack of excitation could cause and the apparent appearance of a steady state after about a week does not necessarily indicate that the smooth muscle regulates sensitivity over only this span of time or only to the extent actually seen.

The loss and the recovery of sensitivity seemed to occur along similar time courses, which accords with the findings in series 2, where intermittent symmetrical fighting schedules were used. Intermittent light causes intermediate degrees of sensitivity. This would not be expected if the time course of recovery from darkness were very different from the time course of recovery from light. On the whole, our findings are compatible with the assumption of (1971) that the receptor density on the smooth muscle cells is regulated and with the further assumption that the receptor population involved has a half-life of approximately 12 d. Strong excitation would prevent the formation of new receptors without accelerating the decay of already formed ones, lack of excitation would accelerate the formation of new receptors again without affecting the removal of already formed ones. This concept explains the similarity in the time course of sensitivity increase and decrease, it is the time course of receptor removal which governs the time constant of both observable processes.

Bitó (1971) has pointed out that choline esters and pilocarpine seem to act on different populations of receptors. Since the present experiments were all done with pilocarpine as an agonist, it is too early to predict if the time course for the other group is similar.

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Inhibition of vasopressin-release during developing hypernatremia and plasma hyperosmolality: An effect of intracerebroventricular glycerol

By

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Abstract

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Non-hydrated goats prolonged (3 h, 0.02 ml/min) intracerebroventricular (IVT) infusion of 0.35 M glycerol depressed the plasma vasopressin level during the entire infusion period which resulted in a compensatory water diuresis exceeding the infusion by about 20 mm. Since no compensatory drinking occurred during the infused water diuresis it gradually induced pronounced dehydration (loss of > 1 liter of total body water causing 5% increase in plasma $[Na^+]$ and osmolality). The same degree of dehydration was induced experimentally by water deprivation. It then caused 5-fold increase in plasma vasopressin.

Concomitant IVT infusions of 0.35 M d-glucose depressed plasma vasopressin level only during the first half of the 3 h infusion period. Consequently the resulting water diuresis was transient and subsided when the glucose infusion was finished.

Plasma osmotic activity increased during the IVT glycerol infusion and during water deprivation, but was fully nullified by IVT glucose. Both IVT glycerol and glucose decreased renal sodium excretion.

The results indicate that the pronounced ability of IVT glycerol to depress the vasopressin release is due not only due to diuresis induced reduction of CSF $[Na^+]$, but also to an influence of glycerol on the vasopressin-releasing mechanism.

Recent evidence indicates that receptors close to the anterior wall of the third cerebral ventricle seem to play an essential role in the regulation of antidiuretic hormone (ADH) release and water intake (cf. Andersson and Olsson 1977). Among the evidence for this concept are the observations that lowering of the $[Na^+]$ of the cerebrospinal fluid (CSF) by intracerebroventricular (IVT) infusions of non-electrolyte solutions induce a water diuresis in non-hydrated, normovolemic goats (Eriksson 1974, 1976), and attenuate dehydrative drinking (Olsson 1975). It was recently found that 1 h IVT infusions of glycerol are much more effective in these respects, and in lowering CSF $[Na^+]$ than the corresponding infusions of

d-glucose (Olsson, Larsson and Liljekvist 1976). However, no quantitative determination of the effect on the ADH release (plasma Arginine Vasopressin - AVP) has been made in previous experiments involving IVT infusions of non-electrolyte solutions. This made it of interest in the present study to follow the effects on water balance and AVP-release of prolonged (3 h) IVT glycerol and d-glucose infusions.

A positive correlation between CSF $[Na^+]$ and renal sodium excretion has been demonstrated in several species (cf. Andersson 1977), whereas changes in CSF $[Na^+]$ are reported to have the reverse effect on plasma renin activity (PRA) (Mourw and Vander 1970, Malm et al. 1974, Eriksson and Fyhrquist 1976). Therefore, the present study was extended to include also determinations of PRA and renal electrolyte excretion.

Methods

Animals: 5 female goats (b.wt. 30–45 kg) were used. They were housed in individual metabolism cages with free access to water except in dehydration experiments. The goats received hay *ad lib.* and were each per day given 6 g of NaCl in 300 g of commercial grain mix.

Implantations and infusions into the lateral cerebral ventricle: All goats had a three-cannula system permanently implanted into one of the lateral ventricles with the ventricular outlet near the foramen of Monro. The cannula material was platinum-iridium in order to minimize foreign body reaction. The construction of the cannula system and the infusion technique have been described earlier (Ålerstedt, Andersson and Olsson 1973). Major advantages of the infusion technique employed were that no dead space added to the system, and that the infusions mixed freely with the ventricular CSF and that the infusions could be started and stopped without the animals being aware of it. Furthermore, samples of CSF could be obtained for analyses in the cannula system used.

Sampling of CSF: Collection of CSF samples were made as previously described (Olsson et al. 1976) before, and 20 min after the infusions. About 0.5 ml of CSF was collected in glass tubes each time for analyses of Na^+ , K^+ and osmolality. The removal of this relatively small amount of CSF did not cause any visible disturbance of the animals.

Sampling of blood: Blood samples were obtained in Brauer-type cannula inserted into the jugular vein. Blood for hormone radioimmunoassay was collected in ice-chilled centrifuge tubes with 0.3 M EDTA as anticoagulant. The blood was immediately centrifuged at $+4^\circ C$ and the plasma was stored at $-20^\circ C$ until radioimmunoassayed. Blood samples for determinations of plasma Na^+ , K^+ and osmolality were collected in heparinized tubes.

Sampling of urine: Urine was collected via a Foley catheter inserted into the urinary bladder.

Assay techniques: Radioimmunoassay for plasma AVP was performed with a method previously applied for determination of human plasma AVP (Fyhrquist et al. 1976 b). The antiserum used was prepared in a rabbit using rainbow-vasopressin as a hapten and bovine thyroglobulin as a carrier. Cross-reactivity with oxytocin (Sandoz, Basel) was $<0.03\%$, with pressinonic acid (Ferring, Malmö) $<0.1\%$, with C-terminus tripeptide of vasopressin (Ferring) $<0.01\%$. Sequential saturation and the use of the Sigma VII preparation (Sigma, St. Louis, Missouri) for standards and preparation of ^{125}I -labelled tracer lowered the assay sensitivity to 0.2 pg/ml. Recovery of synthetic AVP added to pooled goat plasma was 67–93% in the low range (2.5 pg/ml added) and 82–105% in the high range (28 pg/ml added).

Radioimmunoassay of PRA was modified for assay of goat PRA according to Fyhrquist et al. (1974). The optimal pH of angiotensin I generation in goat plasma was found to be 6.2. Therefore, samples were incubated at pH 6.2, in the presence of OH-quinoline 3.5 mM, and Na-EDTA 15 mM. There was no change of pH during the 2 h incubation at $+37^\circ C$. Radioimmunoassay sensitivity was 2.5 pg angiotensin I (2.5 ng deviation from zero dose of unlabelled). Inter-assay variation was 8.5%, intra-assay variation 5.8%.

CSF plasma and urine Na^+ and K^+ were measured by internal standard flame photometry using an IL 343 flame photometer. An Adv Instruments Inc. osmometer was used for determination of osmolality of these fluids. All values presented represent mean \pm S.E.

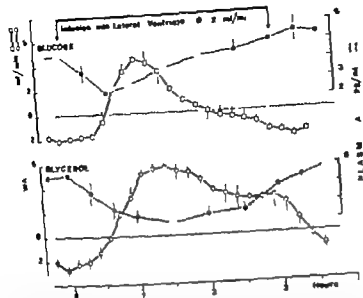


Fig. 1. Effect of 3 h IVT glycerol infusion on water diuresis and AVP release. Top: Water diuresis (ml/min) during IVT infusion of 0.35 M glucose. Bottom: Plasma AVP (pg/ml) during IVT infusion of 0.35 M glucose. The infusion was continued until the end of the infusion period. Number of expts. = 10. Symbols represent mean, vertical bars \pm S.E.

Results

Effects of 3 h IVT glycerol and glucose infusions

Release of AVP and thirst: The IVT infusion of glycerol invariably induced a conspicuous water diuresis in the non-hydrated, normovolemic goat. From pre-infusion respective values, renal free water clearance (C_{H_2O}) turned positive 30–40 min after the onset of the infusion and reached maximal level (5.6 ± 0.5 ml/min) 90 min later. Thus far the effect was identical to that earlier obtained in response to the 1 h infusion (Olsson *et al.* 1976). However, due to the present prolonged IVT administration the water diuresis was sustained for about 3 h, although with some reduction in magnitude (C_{H_2O} about 3 ml/min) after 2 h of infusion (Fig. 1). At this time the animals had become markedly dehydrated as indicated by a rise in plasma [Na⁺] from 148.0 ± 0.7 to 156 ± 0.7 mmol/l. Determinations of plasma AVP showed that this water diuresis was associated with a reduction of plasma AVP levels. Plasma AVP started to decline immediately after the onset of the glycerol infusion, reached minimum value at the time of maximal water diuresis and remained below pre-infusion level as long as renal C_{H_2O} was positive (Fig. 1). Thus, unlike in the water deprivation experiments (see below), there was no correlation between plasma [Na⁺] and plasma AVP values during IVT glycerol infusions (Fig. 2).

Initially the reduction of the plasma AVP level and the development of the water diuresis followed a similar pattern when, instead of glycerol, D-glucose was infused for 3 h into the lateral ventricle. However the IVT administration of glucose was much less effective in

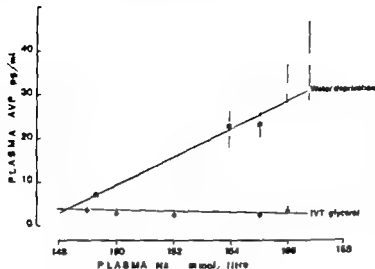


Fig. 2. Scatter. Highly significant correlation ($y = 443.82 + 3.2x$, $r = 0.65$, $p < 0.001$) between plasma AVP values and plasma $[Na^+]$ during dehydration due to water deprivation. Number of experiments = 4. Dots: Lack of such a relation during corresponding stages of dehydration induced by IVT glycerol infusions ($y = 13.87 - 0.08x$, $r = -0.20$). Number experiments = 11. Goats 4. Symbols represent means, vertical bars SE.

maintaining a depression of plasma AVP. Consequently in these experiments, the water diuresis subsided within 2 h of IVT infusion. At this stage plasma AVP had returned to pre-infusion level (Fig. 1).

The IVT administration of glycerol and glucose did not distress the goats or cause behavioural reactions. They were alert and continued to eat and ruminate intermittently during the entire infusion periods. However their urge to drink in response to dehydration was obviously inhibited. In spite of gradually developing dehydration due to excessive urinary water loss, none of the animals drank during the IVT infusions. One of the goats responding to IVT glycerol with the most intense water diuresis, regularly compensated for water loss about 1 h post-infusion. Compensatory drinking was even more delayed in the other animals.

Increase in plasma $[Na^+]$ and osmolality. The water diuresis induced by the 3 h IVT infusion of glycerol resulted in a 620 ± 120 ml net loss of "free" water and a total fluid loss of 1090 ± 180 ml (= about 4% reduction of total body water).

As a result plasma $[Na^+]$ and osmolality increased by 5%. The animals lost considerably less water due to the IVT glucose infusions. Hence, in these experiments the increase in plasma $[Na^+]$ and osmolality was limited to 3%. Plasma $[K^+]$ was not significantly affected by any of the infusions. The data regarding these changes in blood plasma composition are presented in Table I.

Effects on CSF $[Na^+]$, $[K^+]$ and osmolality. Samples of CSF were taken via the cannula system which was used for the IVT infusions. Therefore, no samples could be obtained during the infusion periods and the post infusion sample was not taken until 20 min later, i.e. at a time when the infused solution by all probability had become equilibrated with the CSF of the lateral ventricle. It has been reported previously (Olsson *et al.* 1977) that IVT infusion of glycerol markedly depresses CSF $[Na^+]$. This observation was confirmed in the present study. The 3 h IVT infusion of glycerol induced a fall in CSF $[Na^+]$ from the pre-infusion level of 151 ± 1 to 117 ± 5 mmol/l ($n = 9$) 20 min post-infusion. The fact that the glycerol solution was slightly hypertonic (0.35 M) explains the simultaneous increase in

TABLE I. Changes in plasma $[Na^+]$, $[K^+]$, and osmolality during IVT glycerol (0.35 M) and IVT d-glucose (0.35 M) infusions. The infusion period was 3 h and the rate of the infusion 0.02 ml/min. Note the marked increase in plasma $[Na^+]$ and osmolality at the end of IVT glycerol infusion. Glycerol: -11 points 4; glucose: -10, points -4

	Pre-inf.	Infusion							20 min post-inf.
		20 min	40 min	60 min	90 min	120 min	150 min	180 min	
M_2 mmoles/l Glycerol	148.0 ± 0.7	149.1 ± 0.7	150.0 ± 0.6	152.0 ± 0.6	152.7 ± 0.7	156.0 ± 0.7	154.9 ± 0.9	156.0 ± 0.8	155.9 ± 1.2
Glucose	149.0 ± 0.9	149.4 ± 0.7	150.1 ± 1.0	150.6 ± 1.0	153.0 ± 1.1	153.3 ± 1.1	153.5 ± 1.2	151.6 ± 1.1	152.1 ± 1.5
K^+ mmoles/l Glycerol	4.2 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.0 ± 0.1
Glucose	4.1 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1
osmoles/l Glycerol	296 ± 1	297 ± 1	298 ± 1	300 ± 2	302 ± 1	307 ± 2	306 ± 2	309 ± 2	309 ± 2
Glucose	297 ± 1	299 ± 1	300 ± 1	303 ± 1	304 ± 2	306 ± 1	307 ± 1	304 ± 2	305 ± 2

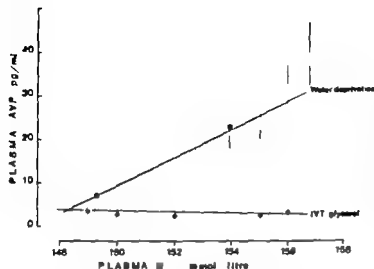


Fig. 2. Scatter: Highly significant correlation ($r = 0.82$, $p < 0.001$) between plasma AVP values and plasma $[Na^+]$ during dehydration due to water deprivation. Number of experiments = 4. Data: Lack of such a relation during corresponding stages of dehydration induced by IVT glycerol infusion ($r = 0.08$, $p > 0.20$). Number experiments = 11 goats. 4 goats represent mean, vertical bars SE.

maintaining a depression of plasma AVP. Consequently in these experiments, the water diuresis subsided within 2 h of IVT infusion. At this stage plasma AVP had returned to pre-infusion level (Fig. 1).

The IVT administration of glycerol and glucose did not distress the goats or cause behavioural reactions. They were alert and continued to eat and ruminate intermittently during the entire infusion periods. However their urge to drink in response to dehydration was obviously inhibited. In spite of gradually developing dehydration due to excessive urinary water loss, none of the animals drank during the IVT infusions. One of the goats responding to IVT glycerol with the most intense water diuresis, regularly compensated a water loss about 1 h post infusion. Compensatory drinking was even more delayed in the other animals.

Increase in plasma $[Na^+]$ and osmolality: The water diuresis induced by the 3 h IVT infusion of glycerol resulted in a 620 ± 120 ml net loss of "free" water and a total fluid loss of 1090 ± 180 ml (= about 4% reduction of total body water).

As a result plasma $[Na^+]$ and osmolality increased by 5 mOsm. The animals lost considerably less water due to the IVT glucose infusions. Hence, in these experiments the increase in plasma $[Na^+]$ and osmolality was limited to 3 mOsm. Plasma $[K^+]$ was not significantly affected by any of the infusions. The data regarding these changes in blood plasma composition are presented in Table I.

Effects on CSF $[Na^+]$, $[K^+]$ and osmolality: Samples of CSF were taken via the same cannula system which was used for the IVT infusions. Therefore, no samples could be obtained during the infusion periods and the post infusion sample was not taken until 20 min later i.e. at a time when the infused solution by all probability had become equilibrated with the CSF of the lateral ventricle. It has been reported previously (Olsson *et al.* 1976) that IVT infusion of glycerol markedly depresses CSF $[Na^+]$. This observation was confirmed in the present study. The 3 h IVT infusion of glycerol induced a fall in CSF $[Na^+]$ from the pre-infusion level of 151 ± 1 to 117 ± 5 mmol/l ($n = 9$) 20 min postinfusion. The fact that the glycerol solution was slightly hypertonic (0.35 M) explains the simultaneous increase in

TABLE I. Changes in plasma [Na], [K], and osmolality during IVT glycerol (0.35 M) and IVT d-glucose (0.35 M) infusions. The 15-min period was 3 h and the rate of the infusion 0.83 ml/min. Note the marked increase in plasma [Na] and osmolality at the end of IVT glycerol infusions. Glycerol, -11 goats; 4; glucose, -10, goats = 4.

	Pre-IVT	Infusion							20 min post-IVT
		20 min	40 min	60 min	90 min	120 min	150 min	180 min	
Na mmol/l									
		149.1 ± 0.7	150.0 ± 0.6	152.0 ± 0.6	152.7 ± 0.7	154.0 ± 0.7	154.9 ± 0.9	156.0 ± 0.8	153.9 ± 1.2
Glycerol		149.4 ± 0.7	150.1 ± 1.0	150.6 ± 1.0	153.0 ± 1.1	153.3 ± 1.1	153.5 ± 1.2	151.6 ± 1.1	152.1 ± 1.5
Glucose									
		4.2 ± 0.1	3.9 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.0 ± 0.1
K mmol/l		4.1 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1
Glycerol									
		296 ± 1	298 ± 1	300 ± 2	302 ± 1	307 ± 2	306 ± 2	309 ± 2	309 ± 3
Glucose		297 ± 1	300 ± 1	302 ± 1	304 ± 2	304 ± 1	307 ± 1	304 ± 2	303 ± 2

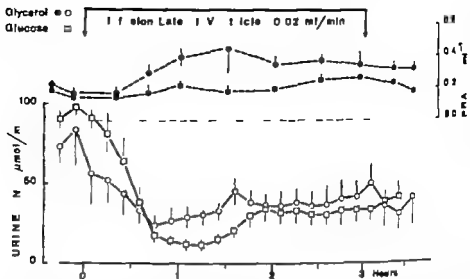


Fig. 3 *Filled symbols*: Increase in PRA during IVT infusions of glycerol (0.35 M) compared to the negligible effect of corresponding glucose infusions. *Open symbols*: Marked depression of renal Na⁺ excretion during both kinds of IVT infusion. Glycerol: Number of expts. = 11 goats = 4. Glucose: Number of expts. = 11 goats = 4. Symbols represent mean, vertical bars S.E.

CSF osmolality from 298 ± 2 to 310 ± 3 mosm/kg. CSF [K⁺] decreased slightly (2.8 ± 0.0 to 2.7 ± 0.0 mmol/l). In comparison the IVT glucose infusion only depressed CSF [Na⁺] to 134 ± 2 mmol/l ($n=8$), while the osmolality increased to 305 ± 1 mosm/kg. The CSF [K⁺] remained unchanged 20 min after the infusion of glucose.

Increase in PRA The IVT infusion of glycerol was accompanied by an increase in PRA which rose from 0.25 ± 0.04 ng ml⁻¹ h⁻¹ prior to the infusion to a peak level of 0.45 ± 0.04 ng ml⁻¹ h⁻¹ after 90 min of infusion (Fig. 3). Thus, PRA reached its highest value at the time when plasma AVP was at its lowest. PRA then declined to about 0.35 ng ml⁻¹ h⁻¹ and this level was maintained throughout the infusion period. Only a minor increase in PRA was observed during the IVT glucose infusion (Fig. 3). PRA rose from 0.18 ± 0.03 to 0.25 ± 0.03 ng ml⁻¹ h⁻¹ within 60 min and continued to fluctuate around this level throughout the infusion period. It had returned to the pre-infusion value 40 min after the infusion.

Decrease in renal Na⁺ and K⁺ excretion. The infusions of both glycerol and glucose caused a reduction in renal Na⁺ excretion (Fig. 3) and to some extent also in renal K⁺ excretion. A decrease in Na⁺ excretion was seen within the first 30 min which became significant after 50 min. After some rise, the Na⁺ excretion was maintained at about 50% of the pre-infusion level throughout the infusion and during the 30 and 40 min post-infusion observation periods. Renal K⁺ excretion showed a similar pattern but the reduction was less pronounced (about 25%).

Effects of water deprivation

The fact that IVT glycerol markedly depressed plasma AVP in spite of developing dehydration made it of interest to study the effects of dehydration to a similar extent under conditions when the CSF composition was allowed to follow the blood plasma changes. In these control experiments drinking water was withheld, and blood, CSF (one animal)

Changes in plasma parameters during 55 h of water deprivation and after rehydration. No. of goats = 4.

AVP pg/ml	PRA ng ml ⁻¹ h ⁻¹	Na mmol/l	K mmol/l	Osmolality mosm/kg
7.21 ± 1.78	0.12 ± 0.02	149.3 ± 0.3	4.2 ± 0.2	293 ± 1
6.78 ± 1.44	0.14 ± 0.02	149.7 ± 0.1	4.2 ± 0.2	294 ± 1
9.43 ± 1.28	0.21 ± 0.02	151.4 ± 0.2	4.4 ± 0.1	298 ± 1
15.33 ± 2.75	0.19 ± 0.04	152.7 ± 0.7	4.2 ± 0.1	299 ± 1
23.10 ± 5.92	0.23 ± 0.03	153.9 ± 0.4	4.2 ± 0.2	303 ± 1
23.00 ± 3.36	0.35 ± 0.04	155.2 ± 0.8	3.9 ± 0.1	305 ± 1
31.43 ± 6.42	0.25 ± 0.04	155.9 ± 0.8	3.9 ± 0.1	307 ± 3
37.95 ± 10.83	0.22 ± 0.04	156.7 ± 0.9	4.1 ± 0.1	310 ± 3
8.90 ± 1.37	0.36 ± 0.03	152.8 ± 1.0	3.7 ± 0.1	299 ± 3
6.55 ± 0.88	0.31 ± 0.05	150.2 ± 1.1	4.1 ± 0.1	298 ± 2
7.00 ± 0.83	0.33 ± 0.05	150.1 ± 1.0	4.0 ± 0.1	294 ± 3

samples were taken at intervals until plasma Na⁺ reached 156 mmol/l and plasma osmolality 309 mosm/kg (= the same mean values as recorded at the termination of the control infusions). These values were reached after 55 h of water deprivation, and then animals were given free access to water. The gradual changes in the composition of the plasma during the 55 h deprivation period, and the effects of rehydration are shown in Table II. In the single animal studied, CSF [Na⁺] had increased from 151 to 161 mmol/l and CSF osmolality from 295 to 312 mosm/kg during the 55 h of water deprivation. Ninety minutes after the animal had satisfied its thirst, the corresponding CSF values were 156 mmol/l and 305 mosm/kg.

Effect of the AVP-releaser: As could be expected from similar studies in man (Robertson et al. 1976), plasma AVP gradually rose during water deprivation concomitant with the rise in plasma [Na⁺] and osmolality. In Fig. 2 is shown the highly significant relation between the increases in plasma [Na⁺] and the rises in plasma AVP which were observed during dehydration induced by water deprivation. For comparison is shown the total lack of such relation at corresponding stages of dehydration during the 3 h IVT infusion of glycerol. There was no rise in PRA. PRA rose during the first 50 h of water deprivation (Table II). It then fell during the remaining 5 h of continued dehydration. However 30 min after that the animals had drunk to satiety there was a steep rise in PRA, and this high level was maintained also in samples taken 60 and 90 min after rehydration.

Effect of Na⁺ and K⁺ excretion: Dehydration induced by water deprivation did not seem to affect the renal Na⁺ excretion which remained between 90 and 125 µmol/min for the whole period. The renal K⁺ excretion fell from a level of about 250 µmol/min during the first 5 h to about 130 µmol/min during the last 5 h of water deprivation. The K⁺ excretion rose to the lower level also for at least 90 min after rehydration. It appears probable that reduced food consumption was the reason for the depressed renal K⁺ excretion towards the end of the dehydration period.

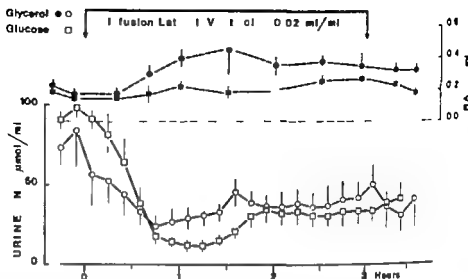


Fig. 3 Filled symbols: Increase in PRA during IVT infusions of glycerol (0.35 M) compared to the marked effect of corresponding glucose infusions. Open symbols: Marked depression of renal Na^+ excretion during both kinds of IVT infusion. Glycerol: Number of expts. = 11 goats = 4. Glucose: Number of expts. = 11 goats = 4. Symbols represent mean, vertical bars S.E.

CSF osmolality from 298 ± 2 to 310 ± 3 mosm/kg. CSF $[\text{K}^+]$ decreased slightly (2.8 ± 0.1 to 2.7 ± 0.0 mmol/l). In comparison the IVT glucose infusion only depressed CSF $[\text{Na}^+]$ to 134 ± 2 mmol/l ($n=8$) while the osmolality increased to 305 ± 1 mosm/kg. The CSF $[\text{K}^+]$ remained unchanged 20 min after the infusion of glucose.

Increase in PRA The IVT infusion of glycerol was accompanied by an increase in PRA which rose from 0.25 ± 0.04 ng ml $^{-1}$ h $^{-1}$ prior to the infusion to a peak level of 0.45 ± 0.04 ng ml $^{-1}$ h $^{-1}$ after 90 min of infusion (Fig. 3). Thus, PRA reached its highest value at the time when plasma AVP was at its lowest. PRA then declined to about 0.35 ng ml $^{-1}$ h $^{-1}$ and this level was maintained throughout the infusion period. Only a minor increase in PRA was observed during the IVT glucose infusion (Fig. 3). PRA rose from 0.18 ± 0.03 to 0.25 ± 0.03 ng ml $^{-1}$ h $^{-1}$ within 60 min and continued to fluctuate around this level throughout the infusion period. It had returned to the pre-infusion value 40 min after the infusion.

Decrease in renal Na^+ and K^+ excretion The infusions of both glycerol and glucose caused a reduction in renal Na^+ excretion (Fig. 3), and to some extent also in renal K^+ excretion. A decrease in Na^+ excretion was seen within the first 30 min which became significant after 50 min. After some rise, the Na^+ excretion was maintained at about 50% of the pre-infusion level throughout the infusion and during the 30 and 40 min post infusion observation periods. Renal K^+ excretion showed a similar pattern but the reduction was less pronounced (about 25%).

Effects of water deprivation

The fact that IVT glycerol markedly depressed plasma AVP in spite of developing dehydration made it of interest to study the effects of dehydration to a similar extent under conditions when the CSF composition was allowed to follow the blood plasma changes. In these control experiments drinking-water was withheld and blood, CSF (one animal)

TABLE II. Changes in plasma parameters during 55 h of water deprivation and after rehydration. No. of pairs 4.

	AVP pg/ml	PRA ng·ml ⁻¹ ·h ⁻¹	Na mmol/l	K mmol/l	Osmolality mosm/kg
30 min after water deprivation (h)					
	7.28 ± 1.78	0.12 ± 0.02	149.3 ± 0.3	4.2 ± 0.2	293 ± 1
	6.78 ± 1.44	0.14 ± 0.02	149.7 ± 0.1	4.2 ± 0.2	294 ± 1
	9.61 ± 1.28	0.21 ± 0.02	151.4 ± 0.2	4.4 ± 0.1	298 ± 1
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	21.10 ± 5.92	0.23 ± 0.03	153.9 ± 0.4	4.2 ± 0.2	303 ± 1
	21.00 ± 3.36	0.35 ± 0.04	155.2 ± 0.8	3.9 ± 0.1	305 ± 1
	31.43 ± 6.42	0.25 ± 0.04	155.9 ± 0.8	3.9 ± 0.1	307 ± 3
5	37.95 ± 10.83	0.22 ± 0.04	156.7 ± 0.9	4.1 ± 0.1	310 ± 3
30 min after rehydration (h)					
1	8.50 ± 1.57	0.36 ± 0.03	152.8 ± 1.0	3.7 ± 0.1	299 ± 3
5	6.55 ± 0.84	0.31 ± 0.03	150.2 ± 1.1	4.1 ± 0.1	296 ± 2
5	7.00 ± 0.85	0.33 ± 0.03	150.1 ± 1.0	4.0 ± 0.1	294 ± 3

plasma samples were taken at intervals until plasma Na⁺ reached 156 mmol/l and plasma osmolality 309 mosm/kg (= the same mean values as recorded at the termination of the IVT glycerol infusions). These values were reached after 55 h of water deprivation, and then the animals were given free access to water. The gradual changes in the composition of the body fluids during the 55 h deprivation period, and the effects of rehydration are shown in Table II. In the single animal studied, CSF [Na⁺] had increased from 151 to 161 mmol/l and CSF osmolality from 295 to 312 mosm/kg during the 55 h of water deprivation. Ninety min after that the animal had satisfied its thirst, the corresponding CSF values were 156 mmol/l and 305 mosm/kg.

Stimulation of the AVP-release. As could be expected from similar studies in man (Robertson and Aicher 1976), plasma AVP gradually rose during water deprivation concomitant with the increase in plasma [Na⁺] and osmolality. In Fig. 2 is shown the highly significant relation between increases in plasma [Na⁺] and the rises in plasma AVP which were observed during dehydration induced by water deprivation. For comparison is shown the total lack of such a correlation at corresponding stages of dehydration during the 3 h IVT infusion of glycerol. **Increases in PRA.** PRA rose during the first 50 h of water deprivation (Table II). It then declined during the remaining 5 h of continued dehydration. However 30 min after that the animals had drunk to satiety there was a steep rise in PRA, and this high level was maintained also in samples taken 60 and 90 min after rehydration.

Renal Na⁺ and K⁺ excretion. Dehydration induced by water deprivation did not seem to affect the renal Na⁺ excretion which remained between 50 and 125 μ mol/min for the whole 55 h period. The renal K⁺ excretion fell from a level of about 250 μ mol/min during the first 30 h to about 130 μ mol/min during the last 5 h of water deprivation. The K⁺ excretion remained at the lower level also for at least 90 min after rehydration. It appears probable that reduced food consumption was the reason for the depressed renal K⁺ excretion towards the end of the dehydration period.

Discussion

A continuing uncompensated loss of body water leads to absolute dehydration. This condition is characterized by hyperosmolality and reduced volume of all body fluid compartments and by elevated extracellular $[Na^+]$. The main compensatory mechanisms brought into play during absolute dehydration are increased release of ADH and thirst with the dual aim to prevent further unnecessary renal waste of water and to restore the water deficit by drinking. It has become generally accepted that the interaction between an "osmotic" and a "volume" regulation governs mammalian ADH-release and water intake. Verney's (1947) ingenious studies conclusively demonstrated that cerebral receptors in the hypothalamic region are involved in the regulation of the ADH-release and implied that these osmoreceptors primarily are excited by changes in the composition of the extracellular fluid which lead to reduction of the receptor volume. This osmoreceptor concept was extended to involve also the regulation of water intake when it was shown that injections of hypertonic NaCl in the anterior hypothalamus elicited excessive drinking in water-replete animals (Andersson 1953). What concerns the "volume" control of water balance, considerable evidence favours the concept that distension receptors in the cardio-vascular system exert a reflex inhibitory tonus of the neurohypophyseal release of ADH (cf. Gauer, Henry and Beim 1970). In addition the renal renin-angiotensin system appears to form an important humoral link in the "volume" regulation of thirst and ADH release (cf. Fitzsimmons 1972).

Paradoxically the osmoreceptor concept eliminates in a strict sense the distinction between "osmotic" and "volume" control of water balance since hypothalamic "osmoreceptors" are thought to monitor intracellular fluid volume. Studies performed predominantly in the goat over the past several years strongly suggest that, instead of osmoreceptors, juxta-ventricular sodium sensitive receptors play an essential role in the regulation of ADH-release and thirst (cf. Andersson and Olsson 1977). This novel idea reverts to a distinction between a regulation influenced by a solute composition of the body fluids on the one hand, and "volume" (= extracellular) regulation on the other. On the whole, the experiments reported here provide further support for this idea. In the present study the same goats were dehydrated to a similar extent in two different ways: a) by water deprivation, and b) by rapidly losing a large amount of body water in response to IVT glycerol. In the first case dehydration induced the appropriate compensatory mechanisms (thirst and accelerated ADH release), in the second case not. It clearly demonstrates that even pronounced changes in the blood of the kind which via cerebral receptors normally stimulate ADH-release and thirst can be made ineffective by IVT infusions of glycerol. This indirectly supports the view (cf. Andersson 1971) that the choroid plexus/CSF is a route by which blood-borne stimuli affect cerebral mechanisms involved in the control of water balance. At the same time, however, the prolonged IVT infusions of glycerol and glucose indicate that the receptors are also affected more directly by the composition of the blood plasma. In spite of maintained subnormal $[Na^+]$ of the CSF the plasma AVP had started to rise again towards the end of the infusion period. It suggests that intense blood-borne stimuli and/or hypovolemic factors may overcome inhibitory influences from the CSF. Ablation experiments (Andersson, Leksell and Libaiko 1975) suggest that the cerebral receptors which lead to ADH-release

at first predominantly are located in the surroundings of the anterior portion of the third ventricle. Since circumventricular structures there are devoid of a blood brain barrier (cf. *Reid and Joynt 1972*), sensory elements in that area may be easily accessible to both local- and CSF-borne stimuli. The experiments reported here confirm and extend the previous observations (*Olsson et al. 1976*) that IVT glycerol lowers CSF $[Na^+]$ and inhibits ADH-release and thirst more effectively than does IVT glucose. It is in agreement with the concept that a juxtaventricular sodium sensitive mechanism is involved in the control of water balance. However, it leaves open questions such as a) why do the two substances have such differing effects upon CSF $[Na^+]$? b) do factors other than mere reduced CSF $[Na^+]$ contribute to the remarkable efficiency of IVT glycerol as inhibitor of ADH release and thirst? Choroidal Na^+-K^+ -ATPase activity is a prerequisite for normal Na^+ transport from the blood to the CSF (*Vates et al. 1964*), and glycerol is an *in vitro* inhibitor of that enzyme system in *Electrophorus electricus* (*Mayer and Avi-Dor 1970*) and in *ox brain* microsomes (*Albers and Koval 1972*). Therefore, reduced entrance of Na^+ into the CSF via the choroid plexus of the lateral ventricle may explain why CSF $[Na^+]$ was 17 mmol/l lower after the IVT infusions of glycerol than after the corresponding glucose infusions. It appears less likely that choroidal Na^+ -coupled glycerol transport out of CSF would have contributed to the observed difference, since transport of that kind would be expected to affect glucose (*Bradbury and Brändstedt 1973*) to at least the same extent as glycerol. However, a contributing factor may also have been glycerol-induced inhibition of periventricular enzymatic Na^+ transport elsewhere than in the choroid plexus. Reduced pumping of Na^+ out of cells surrounding the ventricles may have increased the net diffusional transport of Na^+ from the CSF. If so, the effective stimulus of juxtaventricular sodium sensitive receptors could hardly be increased intracellular Na^+ *per se*. It rather suggests that local Na^+-K^+ -ATPase activity may be of crucial importance for excitation of "sodium receptors" involved in the control of water balance.

Some support for this very hypothetical speculation is provided by the observation that IVT infusions of dextrose (with NaCl added to isotonicity), like glycerol, effectively inhibit ADH-release and thirst in the goat (*Leckell, Lisajko and Rundgren 1976*). Dextrose has also been shown to inhibit Na^+-K^+ -ATPase (*Ahmed and Foster 1974*).

Arginine vasopressin (AVP) is the endogenous ADH in the goat (cf. *Valtin, Stewart and Solod 1974*). That the radioimmunoassay method employed for the present AVP determination (*Fyhrquist et al. 1976 b*) gives a reliable index of the neurohypophyseal ADH-release is evidenced by the highly significant relation between plasma $[Na^+]$ and AVP values which was obtained during the developing dehydration induced by water deprivation (Fig. 2). Hence, this control study supports the assumption that the observed fall in plasma AVP during IVT infusion of glycerol and glucose was due to an inhibition of the basic ADH-release normally going on in the non-hydrated, normovolemic animal.

Artificially induced elevations of the CSF $[Na^+]$ have been found to increase renal Na^+ excretion in many species, which suggests that a juxtaventricular sodium sensitive system also operates in the regulation of renal Na^+ output. In consistence with this idea is the observation in the anesthetized dog (*Mourw and Vander 1970*) and the conscious sheep (*Mourw et al. 1974*) that perfusion of the cerebroventricular system with artificial CSF of low Na^+

reduces renal Na⁺ excretion. However in this respect previous 1 h IVT infusions of various non-electrolyte solutions have given inconclusive results in the goat. In the nonhydrated animal such infusions were not found to induce any consistent change in renal electrolyte excretion (Eriksson 1974), whereas in the hydrated goat the 1 h IVT infusion of fructose caused a fall in the renal K⁺ excretion, indicating a decreased sodium load in the distal tubule in the kidney (Eriksson and Fyhrquist 1976). However in the present experiments a significant reduction in renal Na⁺ excretion was obtained in response to the IVT infusion of glycerol and glucose. It indicates that also in the goat a lowering of CSF [Na⁺] affects renal Na⁺ output in negative direction, and supports the view that juxtaventricular sodium sensitive receptors participate in the control of sodium homeostasis.

In previous studies it has been found that lowering of CSF [Na⁺] causes a rise in PRA (Mouw and Vander 1970; Mouw *et al.* 1974) whereas IVT infusions of hypertonic NaCl depress PRA (Eriksson and Fyhrquist 1976). It has led to the suggestion that also the activity of the renal renin-angiotensin system is under influence of a sodium sensitive cerebral mechanism. That the present IVT infusions, especially of glycerol (causing most pronounced reduction of CSF [Na⁺]) induced a rise in PRA before noticeable dehydration had developed appears consistent with that idea.

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Tension and cyclic GMP changes in potassium depolarized rabbit colon muscle

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Abstract

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Potassium depolarization of rabbit colon muscle elicited a contraction consisting of 2 distinct phases, an initial rapid phasic contraction and a tonic contraction. The tonic contraction was, in contrast to the phasic contraction, dependent on the extracellular calcium for its development. There was a correlation between the tension development and the increase of the cyclic GMP level in the K^+ -depolarized muscle. Experimental conditions which abolished the tonic contracture, i.e. glucose omission and treatment with Ca^{2+} antagonists (verapamil, SKF 525A) also inhibited the cyclic GMP response. The changes of the cyclic GMP levels were Ca^{2+} -dependent. K^+ ions also changed the cyclic AMP content in an effect which was tropine sensitive. From the experimental data obtained in this investigation we suggest that the co-ordination of the tension and the cyclic GMP level in the depolarized colon muscle might depend on oscillations in a common intracellular factor, probably Ca^{2+} .

The mechanisms by which various stimulating agents initiate contractile responses in smooth muscles appear to differ. Edman and Schild (1961), Briggs (1962) and Hurwitz *et al.* (1967) reported that potassium ions initiate a contraction by inducing the influx of extracellular calcium. Acetylcholine, on the other hand, seems to influence calcium compartments in the smooth muscle membrane, and it causes a rapid release of calcium from both superficial and sequestering calcium sites in the membrane.

The importance of cyclic nucleotides in regulating Ca^{2+} -movements in smooth muscle has been discussed by Andersson and Nilsson (1976). The relationship between cyclic nucleotides and contractions elicited by cholinergic drugs has been studied in intestinal smooth muscle (Andersson 1972, Andersson *et al.* 1975, Lee *et al.* 1972). It was found that these drugs first reduced cyclic AMP levels, but later increased the content of the cyclic nucleotide. The content of cyclic GMP was constantly raised after stimulation by cholinergic drugs. Potassium ions have been shown to increase the cyclic AMP content of rabbit colon (Andersson 1972) in ductus deferens of the rat KCl also increased the cyclic GMP level, an effect which was due to the presence of Ca^{2+} ions (Schultz *et al.* 1973).

In this study the relationship between mechanical effects induced by K^+ ions and effects

The cyclic nucleotide levels in colon muscle has been studied. The effect of drugs (verapamil, SKF 525A) that influence the transmembrane Ca^{2+} influx into smooth muscle cells and potassium induced depolarization has also been investigated.

Methods

Smooth muscle employed in this study was isolated from rabbit colon as described by Anderson and Wine-Landstein (1970). Segments approximately 2 cm long and 1 cm wide were fixed into plastic holders and suspended in an organ bath containing 30 ml of Krebs buffer solution. The solution was bubbled with 95% O_2 -5% CO_2 and the temperature of the bath was maintained at 37°C. After 60 min of preincubation, some of the muscles were immersed in K^+ -rich medium. In the experiments where the preparations were depolarized with K^+ -ions, the composition of the suspension solution in mM was: 124.7 KCl, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 , 11.3 NaHCO₃ and 11.5 glucose. In order to deprive the muscles of their Ca^{2+} they were washed repeatedly in calcium-free Krebs solution containing 0.03 mM EGTA, except in the last wash where EGTA was omitted from the solution. In the experiments where the Ca^{2+} antagonists (verapamil and SKF 525A) were used the muscles were preincubated with these drugs for 10-15 min before they were transferred to K^+ -rich medium.

The preparations used for the determination of the metabolic parameters were pretreated with atropine (10^{-6} M) for 10 min, in order to prevent cyclic nucleotide changes due to the liberation of adrenergic muscle substances. For the determination of cyclic nucleotides the tissues were frozen in methanol, then 12% (Boehr) containing dry ice. The frozen muscle preparations were homogenized in 2 ml 5% TCA. After centrifugation the supernatants were adjusted to pH 7.0, and were then charged on to columns AG-1X3 (200-400, borosilicate latex). The cyclic nucleotides were eluted with formic acid. After lyophilization the cyclic AMP content was determined according to Gilman (1970) and cyclic GMP according to Smith *et al.* (1972). The ATP content of the preparations was determined according to Lasepeck *et al.* (1970).

Results

Effects of K^+ ions on cyclic nucleotide levels

After 60 min of preincubation with normal Krebs buffer solution, the mean concentrations of cAMP and cGMP in rabbit colon were 620 ± 50 and 76 ± 7.0 pmol/g wet weight (Fig. 1), respectively. When the muscles were transferred to a K^+ -rich solution a rapid increase of cyclic AMP was observed and a significant rise of cyclic GMP was observed within 30 s and 2 min after transferring them to a K^+ -rich buffer (Fig. 1), the content had nearly tripled. Cyclic AMP levels were reduced by about 25% after 30 s ($p < 0.05$), but increases of this nucleotide (Fig. 1) were observed after both 2 min and 5 min periods. In order to exclude the effect of acetylcholine released from cholinergic nerves by K^+ -ions, the effect of K^+ -ions was also investigated on atropinized muscles. In these preparations K^+ -ions still produced a contraction and increased cyclic GMP but the decrease of the cyclic AMP level observed after 30 sec of incubation with K^+ -ions was absent in atropinized muscles (Fig. 2).

Effects of verapamil and SKF 525A on K^+ -induced contraction and nucleotide changes

The potassium-induced contraction in rabbit colon can be divided in a rapid phasic and a slow tonic component (Fig. 3). The main effect of Ca^{2+} -antagonists like verapamil and SKF 525A is to block the tonic component of the contraction. In higher concentrations also the phasic contraction is completely blocked (Fig. 3 a, b).

The basal levels of cyclic nucleotides

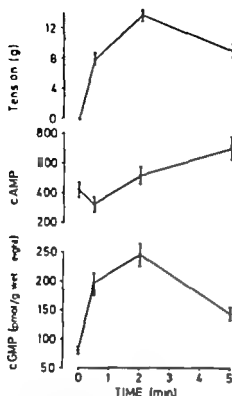


Fig. 1 Influence of K^+ -ions (126 mM) on tension and cyclic nucleotide levels in rabbit colon. Mean \pm S.E. ($n = 10$)

pamil or SKF 525A. However, SKF 525A in a concentration of $1 \cdot 10^{-4}$ g/ml completely blocked both the contraction and the increase of cyclic AMP and cyclic GMP caused by K^+ -ions (Table I). Verapamil ($2 \cdot 10^{-4}$ g/ml) reduced the K^+ -contraction as well as the potassium-induced changes of cyclic nucleotides (Table II). In higher concentrations (10^{-3} g/ml) verapamil completely blocked both contraction and nucleotide changes observed after incubation in K^+ -rich solution.

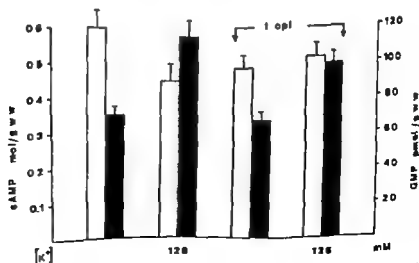


Fig. 2 Cyclic AMP (open bars) and cyclic GMP (filled bars) levels in rabbit colon after incubation in K^+ -high Krebs solution for 30 s in the presence and the absence of atropine (10^{-6} g/ml). Mean \pm S.E.

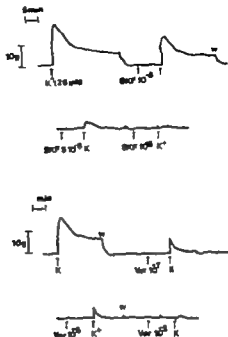


Fig. 1. Effects of different doses (μM) of a) SKF 525A and b) verapamil (Ver) on K⁺ (126 mM) induced contractions of colon muscle. The muscle was washed with Krebs solution at W.

Effect of Ca²⁺ omission and re-addition on tension and cyclic nucleotide content

As illustrated in Fig. 3 the mechanical response of colon muscle to K⁺-rich solution consisted of two phases, a rapid phasic and late tonic contraction. When the concentration of calcium was varied in the incubation solution from 0 to 9 mM the appearance of the potassium contraction changed. The amplitude of the phasic component was rather constant when the calcium concentration was varied in the incubation medium (Fig. 4). The tonic contractions disappeared completely when calcium was omitted from the solution. As is depicted in Fig. 4 there was a gradual improvement of tonic contraction when the calcium concentration in the medium increased up to 9 mM.

The omission of Ca²⁺ from the physiological buffer solution and the deprivation of Ca²⁺ from the muscles with EGTA treatment reduced the cyclic GMP as well as the cyclic AMP

TABLE 1 Effects of K⁺-free (126 mM, 2 min) and SKF 525A (10^{-6} g/ml, 15 min) alone or in combination with each other on tension and cyclic nucleotides in rabbit colon muscle. Mean \pm S.E. 6-7. Degree of significance of the difference from control values is noted by * $p < 0.05$, ** $p < 0.01$.

	cAMP $\mu\text{mol/g w w}$	cGMP $\mu\text{mol/g w w}$	Tension (g)
Control	360 ± 67	34 ± 5	—
K ⁺ -free	$490 \pm 62^*$	$197 \pm 33^{**}$	$13.9 \pm 1^{**}$
SKF 525A	400 ± 60	25 ± 7	—
SKF 525A + K ⁺ -free	310 ± 30	33 ± 4	—

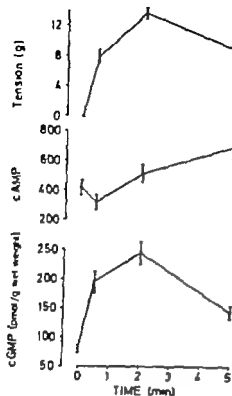


Fig. 1 Influence of K^+ ions (126 mM) on tension and cyclic nucleotide levels in rabbit colon. Mean \pm S.E. (n = 10)

pamil or SKF 525A. However SKF 525A in a concentration of $1 \cdot 10^{-4}$ g/ml completely blocked both the contraction and the increase of cyclic AMP and cyclic GMP caused by K^+ ions (Table I). Verapamil ($2 \cdot 10^{-4}$ g/ml) reduced the K^+ -contraction as well as the potassium induced changes of cyclic nucleotides (Table II). In higher concentrations (10^{-4} g/ml) verapamil completely blocked both contraction and nucleotide changes observed after incubation in K^+ -rich solution.

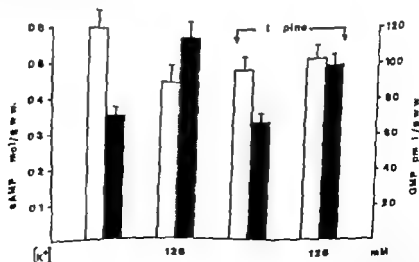


Fig. 2 Cyclic AMP (open bars) and cyclic GMP (filled bars) levels in rabbit colon after incubation in K^+ -high Krebs solution for 30 s in the presence and the absence of tropine ($1 \cdot 10^{-4}$ g/ml). Mean \pm S.E. (n = 6).

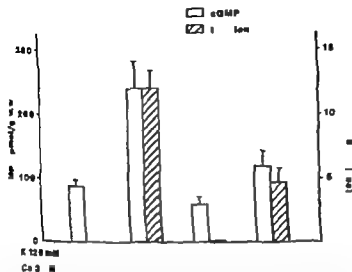


Fig. 5. Influence of Ca^{++} on the effect of K^+ (126 mM) on tension and cyclic GMP levels in colon muscles. Muscles were either preincubated in normal Krebs solution or in Ca^{++} -free medium containing EGTA for 8 min and then transferred for 2 min to fresh medium containing Ca^{++} and K^+ as indicated in the figure. In several instances addition of Ca^{++} alone. Mean \pm S.E. ($n=6$).

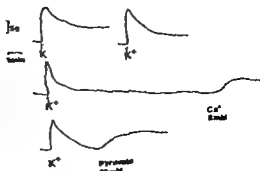
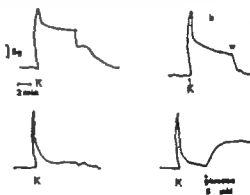


Fig. 6. The effect of removing glucose from the incubation medium on K^+ (126 mM) induced isometric contractions and contractions elicited in the presence of glucose. Contractions elicited 20 min (b and f), and 60 min (c) after the removal of glucose. The addition of glucose (5.5 mM d), Ca^{++} (8 mM g) and pyruvate (10 mM h) to muscles incubated in the absence of glucose for 60 min.

TABLE II Influence of different concentrations of verapamil on potassium (126 mM) induced changes in cyclic nucleotide levels and tension. Mean \pm S.E. (n=6-12). Degree of significance denoted as in Table I

Drugs	Cyclic AMP pmol/g w w	Cyclic GMP pmol/g w w	Tension g
Control	387 \pm 60	21.3 \pm 2.7	—
K ⁺ -ions (126 mM)	539 \pm 75	121.7 \pm 23.4	9.2 \pm 1.0*
Verapamil (2 \cdot 10 ⁻⁴ g/ml)	373 \pm 55	27.3 \pm 6.0	—
Verapamil (2 \cdot 10 ⁻⁴ g/ml) + K ⁺ -ions (126 mM)	340 \pm 63	95.0 \pm 7.8	2.3 \pm 0.3
Verapamil (1 \cdot 10 ⁻⁴ g/ml)	453 \pm 30	11 \pm 6.4	—
Verapamil (1 \cdot 10 ⁻⁴ g/ml) + K ⁺ -ions (126 mM)	468 \pm 52	32.0 \pm 10.7	0.3 \pm 0.1

levels of the muscle. In such muscles, both the contractile and the cyclic GMP elevation effects of K⁺ ions were lost. The readdition of Ca⁺⁺ (3 mM) to this muscle restored the contractile response to K⁺ and also the effects on cyclic GMP and cyclic AMP (Fig. 5).

Effects of glucose omission on tension and cyclic nucleotide content

When glucose was omitted from the incubation medium the tonic contracture induced by K⁺ ions gradually disappeared while the phasic contraction was almost unchanged. After 11 of incubation in a glucose free medium only the phasic contraction could be detected (Fig. 6 c). The tonic contracture could be restored either by carbohydrate metabolites (glucose pyruvate) or by a high concentration of Ca⁺⁺ (8 mM) (Fig. 6 d, g, h). The increase of cyclic GMP observed in the K⁺-depolarized muscle was abolished in the glucose deficient medium (Table III). The levels of cyclic AMP and energy-rich phosphate compounds such as ATP and CrP were also lowered. The addition of glucose or pyruvate to the glucose-deficient muscle restored both the tonic contracture and the cyclic GMP level (Fig. 6 and 7). A similar effect was also demonstrated when of CaCl₂ (8 mM) was added (Fig. 6 and 7). As is evident from Figure 8 an increase of tension in the glucose-deficient K⁺-depolarized muscle is associated with an increase of cyclic GMP. This covariation occurred both with carbohydrates and Ca⁺⁺-ions.

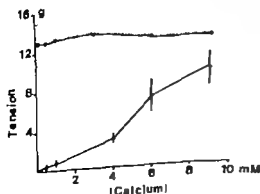


Fig. 4 Effects of different concentrations of calcium in incubation solution on potassium induced contraction of colon muscle. Dotted area amplitude of phasic contraction. Solid line tonic contraction. Mean \pm S.E. (n=8)

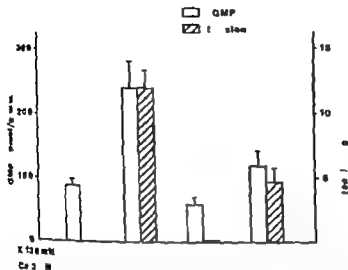


Fig. 5. Effect of K^+ on the effect of K^+ (126 mM) on tension and cyclic GMP levels in colon smooth muscle. The muscle was precontracted in normal Krebs solution or in Ca^{++} -free medium containing EGTA for 2 min and then transferred for 2 min to fresh medium containing Ca^{++} and K^+ as indicated in the figure. Each value is the mean \pm S.E. ($n=6$).

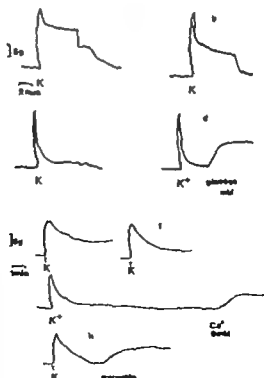


Fig. 6. The effect of removing glucose from the incubation medium on K⁺ (126 mM) induced contractions and cyclic GMP levels. Contractions elicited in the presence of glucose. Contractions elicited 20 min (b and f), and 60 min (d and h) after the removal of glucose. The addition of glucose (5.5 mM) (d, f), Ca^{++} (1 mM) (g) and glucose (5.5 mM) (h) to muscles incubated in the absence of glucose for 20 min.

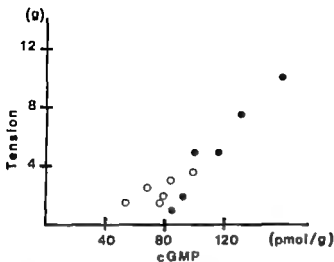


Fig. 7 Effects of Ca^{++} (0 mM open circles) and glucose (5.5 mM closed circles) on the tension and cyclic GMP in colon muscles suspended in a glucose-free Krebs solution for 1 h.

Discussion

The mechanical response of rabbit colon muscle to K^+ -rich Krebs solution consisted of two distinct phases: an initial rapid rise of tension (the phasic response), and an ensuing tonic contraction. A similar effect of high potassium medium has earlier been shown by Darb and Jenkinson (1961), Urakawa and Holland (1964) and Imai and Takeda (1967) on guinea pig taenia coli and by Somlyo and Somlyo (1969) on rabbit mesenteric vein. The tonic component of the contraction seemed to be most sensitive to drugs such as Verapamil or SKF 525A (Fig. 3). Higher concentrations of these drugs also affected the phasic response. Verapamil and SKF 525A have been shown to block transmembrane Ca^{++} influx in smooth muscles (Fleckenstein *et al.* 1969; Haercker 1972; Kalsner *et al.* 1971). Briggs (1963) and Hinke *et al.* (1964) suggested that the K^+ contraction of arterial smooth muscle depends on an influx of Ca^{++} from the extracellular space. According to our experiments we suggest that this interpretation may also apply to the tonic contraction of colon muscle. The degree of tonic contracture was closely related to the extracellular calcium concentration (Fig. 4) while the phasic contraction was almost independent of extracellular calcium in the depolarizing solution.

The present data show that the influx of Ca^{++} is also a very important factor for the regulation of

TABLE III Effects of glucose omission on cyclic nucleotides ATP and CrP levels in polarized and K^+ depolarized colon muscle. The metabolites were determined after an incubation period of 10 min in K^+ -rich Krebs buffer and 2 min after the glucose-addition. Means \pm S.E. $n = 6$. Degree of significance denoted as in Table I

Solutions + additions	Cyclic AMP pmol/g w w	Cyclic GMP pmol/g w w	ATP nmol/g w w	CrP nmol/g w w
Normal Krebs	271 \pm 42	70 \pm 7.0	590 \pm 90	299 \pm 67
K^+ rich Krebs	300 \pm 30	133 \pm 15	555 \pm 50	197 \pm 51
K^+ rich Krebs without glucose	170 \pm 10	88 \pm 2	387 \pm 9	72 \pm 7
K^+ rich glucose + 5.5 mM glucose	210 \pm 20	106 \pm 7	—	—

of cyclic GMP levels in colon muscle. The increase of cyclic GMP observed during K⁺-contraction seemed to be Ca²⁺-dependent, as it was absent in Ca-depleted muscles (Fig. 5) and in muscles where the transmembrane influx of Ca²⁺ was blocked by drugs (Table I; Fig. 4). In ductus deferens and submaximal glands Schultz *et al.* (1973) also found Ca²⁺-dependent increase of cyclic GMP levels by cholinergic agonists and by KCl. These observations suggested that the most probable reason for the cyclic GMP increase was a stimulation of the guanylate cyclase activity by Ca²⁺. Handman *et al.* (1971) have shown that Ca²⁺ is capable of stimulating guanylate cyclase obtained from various tissues. In another study we have also demonstrated an activation of colon muscle soluble guanylate cyclase by high concentrations of Ca²⁺ (Nilsson and Andersson 1977).

The transient decrease of cyclic AMP following a K⁺-contraction could possibly depend on release of the cholinergic transmitter since this effect was blocked by atropine. Moreover, cholinergic drugs have been shown to produce a transient decrease of cyclic AMP levels in rabbit colon (Andersson 1973). Paton and Aboo Zar (1968) showed that K⁺-ions displace acetylcholine from intestinal muscle preparations.

The observations that β -adrenoceptor mediated relaxation are accompanied by elevated cyclic AMP levels (Triner *et al.* 1971; Andersson 1972) and cyclic GMP levels are elevated by contracting agents (acetylcholine, KCl, Lee *et al.* 1972, Schultz *et al.* 1973; Andersson *et al.* 1975) may support a regulatory role of cyclic nucleotides in the contraction-relaxation of smooth muscle. There has often been confusion and controversy as to the relative importance of the cyclic nucleotides in smooth muscles, but cyclic AMP seems to promote relaxation in most smooth muscle and the contraction is mostly associated with increased cyclic GMP levels. It has also been found that cyclic AMP stimulated Ca-binding to smooth muscle microsomes is antagonized by cyclic GMP (Nilsson and Andersson 1977).

Any direct role for cyclic nucleotides in the K⁺-induced contraction seems improbable. The most probable mechanism seems to be that the depolarization caused by K⁺-ions in the cell membrane changes in such a way as to permit the entrance of calcium into the cytoplasm. This has been suggested by Hinke *et al.* (1964) and Hurwitz and Saria (1971). However, the increase of cyclic GMP observed during K⁺-contraction might have a modulating effect on contraction. It could possibly promote maintenance of the tonic contraction. Under conditions which suppress cyclic GMP levels *viz.* glucose omission or calcium antagonism (Fig. 7; Table I-III) only phasic contraction was elicited. The tonic contracture and the increase of cyclic GMP levels in the glucose-free muscles was restored by carbohydrates.

This fact may indicate that glucose or pyruvate are immediately utilized in the development of contracture by depolarized muscles an observation which is in accordance with the results of Anderson *et al.* (1965). An increased tonic contraction in the glucose-free muscle can, however, be obtained by increasing the Ca²⁺-concentration in the extracellular medium (Fig. 6). This indicates that the energy supply to the contractile elements is not limiting factor. The cytoplasmic Ca²⁺-concentration might instead have been too low in the glucose-free muscle in order to activate the contractile proteins and to increase the cyclic GMP content. It is, however, not possible from this study to determine if in the K⁺-depolarized colon muscle, the cyclic GMP and the tension changes are causally related, or if the contraction is dependent on a common intracellular messenger such as Ca²⁺.

We are indebted to Mrs Ingrid Salomonsson, Mrs Eva Hägglund and Mr Sverter Björjesson for assistance. Financial support has been provided by the Swedish State Medical Research Council 64 and Magnus Bergvalls Foundation.

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[5] Mixed Liver function was estimated from the hepatic oxygen uptake, the hepatic clearance of inulin (Cl_{in}) and the hepatic elimination rate of ethanol. The operational techniques, flow measurements, and analytical procedures and calculations have all been described elsewhere in detail (see [17], Knap, Larsson and Månck 1976). Secretin (pure, porcine secretin, O.L.H. Research Unit, Smedby Department, Karolinska Institute, Stockholm) was given i. v. in isotonic NaCl solution, pH 7.2. Indomethacin (Merck, Sharp & Dohme B. V. Haarlem, Netherlands) was dissolved in 90% ethanol and added to 15 ml 0.9% sodium chloride solution containing 150 mg sodium bicarbonate and heated in a water bath at 40°C. The results are given as mean values \pm S.E. and the effects of secretin and indomethacin tested by the Student's *t*-test for paired comparisons.

Results

In results from experiments on 8 cats in which secretin was infused at a rate of 2 U kg⁻¹ h⁻¹ (Table I and II). Infusion of secretin was always followed by an immediate rise in bile flow which stabilized in the second or third 15-min period and remained stable for the rest of the experimental period of up to 2 h. A barely significant rise in erythritol clearance was also noticed. Thus the bile flow which initially was lower than the erythritol clearance fully exceeded it. It appears therefore that the increase in bile flow was caused almost entirely by a change in the ductular net fluid transport, which reversed from a net reabsorption to a net secretion of fluid. The increase in flow was accompanied by an increase in the electrolyte excretion rate of Na⁺, K⁺, Cl⁻ and HCO₃⁻ whereas no change was observed in the excretion rate of bile acids. In 5 cats where liver hemodynamics and liver metabolism were followed, secretin did not affect hepatic arterial blood flow (12.6 \pm 2.0 ml kg⁻¹ min⁻¹), portal blood flow (21.8 \pm 3.9 ml kg⁻¹ min⁻¹), hepatic arterial conductance (0.09 \pm 0.01 ml kg⁻¹ min⁻¹ mmHg⁻¹), portal conductance (3.5 \pm 0.3 ml kg⁻¹ min⁻¹ mmHg⁻¹) or sinusoidal conductance (0.17 \pm 0.03 ml kg⁻¹ min⁻¹ mmHg⁻¹). Neither was any change observed in the hepatic oxygen uptake (45.0 \pm 6.5 μ mol kg⁻¹ min⁻¹), ICG-clearance (4.9 \pm 1.4 ml kg⁻¹ min⁻¹) or hepatic ethanol elimination (29.0 \pm 5.1 μ mol kg⁻¹ min⁻¹). In 8 cats secretin was infused at a rate of 2 U kg⁻¹ h⁻¹ as above. Once bile flow was stable, indomethacin was given i. v. as a single dose of 25 mg kg⁻¹. Results from these experiments are listed in Table I and II. Indomethacin reduced bile flow significantly but this reduction was mainly due to a reduction in canalicular bile production. There was also a slight reduc-

Table I. Effects of secretin and of secretin + indomethacin on bile flow and erythritol clearance. Mean values \pm S.E. \pm 8 expts. before (A) and during (B) infusion of secretin 2 U kg⁻¹ h⁻¹. 11 8 expts. during (C) infusion of secretin 2 U kg⁻¹ h⁻¹ and (D) supplemented with indomethacin 25 mg kg⁻¹.

	I		II	
	A	B	C	D
Bile flow (ml kg ⁻¹ min ⁻¹)				
2-to-plasma ratio of Erythritol	11.4 \pm 0.7	21.1 \pm 0.8***	22.7 \pm 2.0	17.8 \pm 1.9*
Erythritol clearance (ml kg ⁻¹ min ⁻¹)	1.45 \pm 0.08	0.85 \pm 0.04***	0.86 \pm 0.06	0.91 \pm 0.08**
Ductular net fluid transport (ml kg ⁻¹ min ⁻¹)	16.3 \pm 0.8	17.8 \pm 0.6	19.2 \pm 1.6	15.8 \pm 1.4**
	- 4.8 \pm 0.8	+ 3.3 \pm 0.9***	+ 3.3 \pm 1.5	+ 2.0 \pm 1.6**

* $p < 0.05$, ** $0.005 < p < 0.010$, *** $p < 0.001$

The effect of secretin on bile production, splanchnohepatic hemodynamics and liver function in cats

By

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Abstract

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The effect of secretin on bile flow, biliary clearance of ^3C -erythritol and bile composition was studied in fasted, chloralose anesthetized cats. Secretin ($2 \text{ U kg}^{-1} \text{ hr}^{-1}$) increased bile flow by 85% and increased rates of excretion of Na^+ , Cl^- and HCO_3^- . Bile flow increased from values below to values above the critical clearance, which did not change significantly. It is therefore concluded that secretin altered ductular transport of fluid from a net reabsorption to a net secretion of bicarbonate rich fluid. Secretin did not affect splanchnohepatic hemodynamics or the overall function of the liver and indomethacin did not significantly alter the response to secretin.

The choleric effect of secretin is well established in humans as well as in a variety of animals (Erlinger and Dhumeaux 1974) but only few reports deal with the choleric action of secretin in cats (Tanturi, Ivy and Greengard 1937; Scratcherd 1965). It was recently reported (Krarup, Larsen and Munck 1976) that infusion of prostaglandin E_1 and E_2 influenced bile flow in cats in a way which mimicked the effect of secretin in other animals. The present experiments were therefore undertaken with the purpose of examining the choleric action of secretin in cats together with the possible role of prostaglandin in secretin choleresis. Also the effect of secretin on splanchnic hemodynamic parameters and liver metabolism was followed.

Methods

Fasting cats (weight 3.4 ± 0.2 (S.E.) kg) anesthetized with chloralose (50 mg kg^{-1}) and Nembutal (30 mg given i.p.) were used for the experiments. Catheters were placed in the femoral artery and vein, the portal vein and a hepatic vein for blood sampling, infusion and pressure measurements. Flow probes were placed around the portal vein and the hepatic artery and blood flow was measured by an electromagnetic flowmeter (Nycotron, Oslo). Bile was collected from a catheter placed in the choledochus after ligation of the cystic duct. To compensate for the abrupt enterohepatic circulation of bile acids, taurocholate ($0.2 \text{ mmol kg}^{-1} \text{ min}^{-1}$) was infused continuously. The canalicular bile flow was estimated by the biliary clearance of ^3C -erythritol and the net reabsorption/secretion of fluid in the biliary ductules was calculated as the difference between erythritol clearance and bile flow. The biliary excretion rate of bile acids and inorganic ions

more significantly and the results do not therefore support the assumption that the effect of secretin on bile ducts is mediated via prostaglandins.

In our experiments secretin was found to be without effect upon the splanchno-hepatic vascular bed; there are only a few and conflicting reports to be compared with these results. LIND (1970) found that close intraarterial injections resulted in an increase in flow and conductance of the mesenteric arteries in cats, whereas injection of secretin into the hepatic artery was followed by an indirect and delayed decrease in flow and conductance. In contrast to these findings Richardson and Witherington (1976) found that secretin increased conductance and flow in the perfused arterial vascular bed of the dog's liver. The discrepancies between the findings may be due to differences in species, dosage and experimental technique.

In conclusion the present results indicate that the biliary effect of secretin in the cat is mainly if not exclusively localized on the bile ductular epithelium and is not accompanied by secondary to hemodynamic changes or changes in the overall function of the liver.

The skilled technical assistance of Miss Karin Donby is gratefully acknowledged.

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TABLE II Effects of secretin and of secretin + Indomethacin on the rates of excretion of various components. The rates of biliary excretion of anions and cations are given as $\mu\text{mol kg}^{-1} \text{hr}^{-1}$ values \pm S.E. of 8 expts. before (A) and during (B) infusion of secretin $2 \text{ U kg}^{-1} \text{ hr}^{-1}$ II. during (C) infusion of secretin $2 \text{ U kg}^{-1} \text{ hr}^{-1}$ and (D) supplemented with Indomethacin 25 mg

	I		II	
	A	B	C	D
Na	1.74 ± 0.09	3.18 ± 0.13	3.42 ± 0.35	$2.76 \pm 0.30^{**}$
K	0.03 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	$0.08 \pm 0.01^{**}$
Cl^-	1.25 ± 0.11	2.26 ± 0.11	2.44 ± 0.22	$1.87 \pm 0.19^{**}$
HCO_3^-	0.32 ± 0.02	0.82 ± 0.03	0.83 ± 0.14	$0.63 \pm 0.10^*$
Bile acids	0.29 ± 0.01	$0.31 \pm 0.01^{**}$	0.36 ± 0.01	$0.31 \pm 0.03^{**}$

$0.01 < 0 < 0.02$, * $0.001 < p < 0.005$ $p < 0.001$

tion in the net ductular fluid secretion rate and the rates of excretion of electrolytes change in the excretion rate of bile acids. In a few experiments Indomethacin was administered prior to the infusion of secretin the responses to secretin were essentially the same as obtained when no pre treatment was given.

Discussion

The observed effects of secretin on bile flow and composition are similar to those for conscious dogs given the same amount of secretin (Preisig, Cooper and Wheeler 1962, and Grossman 1969 Jones, Geist and Hall 1971 Soloway *et al* 1972, Russell, Searl Jones 1975 and Kaminski, Ruwart and Jellinek 1975). This indicates that the anaesthesia applied is without major importance for the choleretic effect of secretin. The marked effect in ductular net fluid transport, the lack of effect on bile acid secretion as well as the increase in the excretion rate of bicarbonate are all in accordance with the present view on the effect of secretin on bile production. According to this, the main effect of secretin is the secretion of a bicarbonate-rich fluid in bile ductuli (Boyer and Bloomer 1974, Strasberg *et al* 1975). In the present experiments secretin changed a net fluid reabsorption in the ductuli to a net secretion. Correspondingly the bile-to-plasma ratio of erythritol changed from above unity (1.4) to below (0.85). A ratio greater than 1.0 was also found during control conditions in dogs (Wheeler, Ross and Bradley 1968) and rabbits (Erlinger *et al* 1970) whereas a ratio smaller than 1.0 was found in guinea pigs (Forker 1967) and dogs (Erlinger and Dumont 1971) as well as in humans (Prandi *et al* 1975). Boyer (1971) found a value close to 1.0 in the perfused rat liver. These discrepancies may be explained by differences in the nutritive state of experimental animals and thus the secretion rate of secretin. This explanation is supported by the experiments of Strasberg *et al* (1975) on monkeys in which bile-to-plasma ratio of erythritol was greater than 1.0 during fasting and smaller than 1.0 after feeding.

The results demonstrate that the effects of secretin on bile production in the cat are similar to those obtained with prostaglandins (Krurup, Munck and Larsen 1976). However, Indomethacin, which inhibits the synthesis of prostaglandins (Vane 1971 Smith and LaPlante 1971 Alfonso *et al* 1974 and Clark and Cumby 1975) did not affect the ductular response.

Effect of the nonionic detergent Triton X 100 on sodium permeability of the myelinated nerve fibre of *Xenopus laevis*

By

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Abstract

BRISMAR, T. and B. RYDQVIST. Effect of the nonionic detergent Triton X 100 on sodium permeability of the myelinated nerve fibre of *Xenopus laevis*. Acta physiol. scand. 1978. 102. 425-433.

The effect of the nonionic detergent Triton X-100 (TX 100) on the Na permeability (P_{Na}) properties of the node of Ranvier in myelinated nerve fibres of *Xenopus laevis* was analysed with potential clamp techniques. Application of TX-100 caused rapid initial decreases in P_{Na} that was reversible 1 min after as well as the irreversible block. Block effects depended on [TX 100] and duration of exposure. The reversible reduction of P_{Na} at the steady state was 30% at 40-60 μ M TX 100. The slope of the Hill plot for the reaction was 1.75 indicating deviation from first order reaction. The equilibrium dissociation constant (K_D) for TX 100 was $0.9 \cdot 10^{-6}$ (M^{-1}). K_D calculated from the rate constants for onset and offset of the reversible reaction (K_2/K_1) was $1.5 \cdot 10^{-6}$ (M^{-1}). The possibility that the action of TX-100 involves membrane proteins is discussed.

The conduction of impulses in nerve is associated with specific changes in the ionic permeability properties of the membrane (cf Hodgkin 1964). The potential and time relations of the permeability mechanisms have been described for squid giant axons (Hodgkin and Huxley 1952) and myelinated nerve fibres (Frankenhaeuser and Huxley 1964). The structure of the permeability site is still unknown. Some evidence indicate that proteins constitute part of it. β Armstrong, Benzerilla and Rojas (1973) have found that the proteolytic action of trypsin on the inside of squid axons selectively destroys the mechanism for Na permeability activation. Several investigators have tried to characterize chemically the permeability properties on the basis of experiments with substances which affect the permeability properties and have more or less well defined action sites (cf Narahashi 1974).

It has been found that nonionic detergents block the conduction in nerve and the neuro-muscular transmission (Sjostrom and Soehring 1952, Soehring, Deas and Stave 1952, Zipf and Dettmann 1964, Dettmann 1973). The nonionic detergents may interact with biological membranes in several ways. A perturbation of the lipid phase may alter the physical properties of the bilayer. At high concentrations nonionic detergents possibly form mixed

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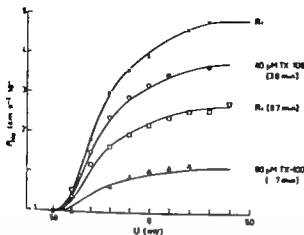


Fig. 1. Effect of TX-100 on peak Na permeability (P_{Na}) vs. potential (U) curve. Membrane polarized to -60 mV between test pulses. P_{Na} calculated from records of membrane current obtained in different external solutions applied in the following order: Ringer solution (R), after 38 min in 40μ M TX 100 (O), after 87 min in Ringer's solution (□), and after 7 min in 80μ M TX 100 (Δ). Axon 1 Temp. 11–13°C.

(Frankenhaeuser 1960, Brismar 1977 b). Experiments with conditioning pulses of 100 ms indicated that TX-100 did not affect the steady-state relation between P_{Na} and membrane potential.

When high concentrations of TX 100 (160μ M in the present experiments) were applied or TX-100 in lower concentrations acted for long durations (> 30 min) then suddenly a large increase in membrane leak current appeared which terminated the experiment. Still lower concentrations TX 100 did not affect the leak and the capacitive current amplitude and did not cause an increased rate of Na entry which generally was associated with the 'run down' of the isolated fibre. The rate of Na entry was monitored by repeated measurements of the Na equilibrium potential. The time course of the action of TX-100 on the peak P_{Na} was obtained from measurements of the change in amplitude of the peak Na current associated with a positive potential pulse applied repeatedly. Fig. 2 shows the time course of the change in peak P_{Na} calculated in this way. The size of the effect was clearly related to the concentration of TX-100 in the external solution and the duration of the exposure. A steady state was reached but the initial phase of decrease in P_{Na} was succeeded by a slow continuous depression. This together with the observed persistence of the reduction in P_{Na} after wash out of TX-100 indicated that TX-100 had a partially irreversible action.

Analysal model of TX 100 action

In order to account for the finding that TX-100 had both a reversible and an irreversible effect on P_{Na} and to provide a basis for a quantitative description it was necessary to put up a hypothetical model for the reaction between TX-100 molecules and acceptor sites on the nodal membrane. It was assumed that the reversible effect was due to a reversible binding of TX-100 molecules on acceptor sites on the nodal membrane, thereby blocking the Na per-

micelles with different phospholipids (Dennis 1974). Recent studies have demonstrated that nonionic detergents can bind to proteins and in particular to more hydrophobic proteins (Mikano Reynolds and Tanford 1973 Simons, Helenius and Garoff 1973 Helenius and Simons 1975). The proteins investigated have been shown to have both high and low affinity sites.

In the present study the action of a nonionic detergent, Triton X 100 (TX 100), on the Na permeability mechanism in myelinated nerve fibres was investigated. Potential clamp experiments were performed on the node of Ranvier in single fibres dissected from the frog (*Xenopus laevis*). TX 100 decreased the Na permeability. The magnitude of its blocking action increased with the concentration and the time of application. The effect of TX 100 was only partially reversible. The results are tentatively described in terms of agonist-receptor kinetics.

Methods

Myelinated nerve fibres were dissected from the sciatic nerve of the frog (*Xenopus laevis*). Single fibres were isolated and mounted in a perspex recording chamber. The potential clamp technique was similar to the earlier described by Dodge and Frankenhaeuser (1954) with minor modifications (Brismar and Frankenhaeuser 1972). Some of the experiments were made in a recording chamber allowing very fast solution changes. The half time for the effective solution change at the nodal membrane was 100–150 ms for pH changes (Brismar 1977). The diffusion of TX 100 into the nodal gap may however be considerably slower than for Na.

TX 100 was purchased from Rohm and Haas, Sweden, and used without further purification. The detergent belongs to a series of homologues with the general formula $C_{18}H_{33}O_2(C_6H_4O)_nH$. The mean molecular weight of TX 100 is 628 (Handbook of Physical Properties, Rohm and Haas Surfactants CS-01a). Triton surfactant agents. The nonionic octylphenoxypolyethanol (OPE) series, C₈-401/d. The TX 100 m is 8 and the mean number of ethyleneoxide groups, n , is 9.8. The commercially available detergent is not homogeneous but a mixture of detergents with a Poisson distribution of n (Sachar and Greet 1967).

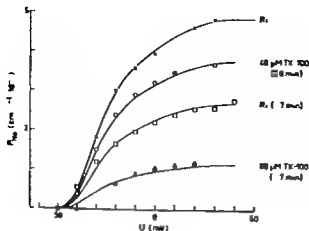
Test solutions were made with various concentrations of TX 100 (40, 60, 80 and 160 μ M). The electrolyte composition was in all test solutions 112 mM NaCl, 2.5 mM KCl, 2.0 mM CaCl₂ and 2.5 mM Tris buffer pH 7.4 (25°C).

Nomenclature. The membrane potential (U) is inside potential minus outside potential. Outward currents are consequently positive.

Results

Effects of TX 100 on Na permeability

The effect of the detergent TX 100 on the Na permeability (P_{Na}) in single myelinated nerve fibres was analysed with potential clamp technique. The membrane currents associated with positive potential pulses were recorded. During exposure to TX 100 the amplitude of the Na current decreased. A slight decrease in the delayed outward potassium current (I_K) was also observed. This latter effect was not studied further. The peak P_N was calculated from the peak Na current and the constant field equation (Dodge and Frankenhaeuser 1959). Fig. 1 shows peak P_N vs. membrane potential (U) before, during and after successive application of 40 and 80 μ M TX 100 in Ringer's solution. A smooth curve was fitted to the symbols representing P_{Na} before TX 100 was applied, and from this curve the other curves were obtained by scaling the ordinates with constant factors. It is evident that TX 100 decreased the available P_{Na} but did not affect the potential dependence of P_{Na} activation. The membrane was negatively polarized to -120 mV between the test pulses, which removed P_{Na} inactivation.



Effect of TX-100 on peak Na permeability (P_{Na}) vs. potential (U) curve. Membrane polarized to 8 mV between test pulses. P_{Na} calculated from records of membrane current obtained in different solutions applied in the following order: Ringer's solution (X), after 3.0 min in 40 μ M TX-100 (O), then in 80 μ M TX-100 (Δ), and after 8.7 min in Ringer's solution (\square). Axon L Temp. 11–13°C.

raizenhacomer 1960, Briamar 1977 b). Experiments with conditioning pulses of amplitudes indicated that TX 100 did not affect the steady-state relation between activation and membrane potential.

high concentrations of TX-100 (160 μ M in the present experiments) were applied or TX-100 in lower concentrations acted for long durations (> 30 min) then suddenly a increase in membrane leak current appeared which terminated the experiment. Still concentrations TX 100 did not affect the leak and the capacitive current amplitude and cause an increased rate of Na entry which generally was associated with the 'run' of the isolated fibre. The rate of Na entry was monitored by repeated measurements of equilibrium potential. The time course of the action of TX 100 on the peak P_{Na} was deduced from measurements of the change in amplitude of the peak Na current associated with a positive potential pulse applied repeatedly. Fig. 2 shows the time course of the change in P_{Na} calculated in this way. The size of the effect was clearly related to the concentration of TX-100 in the external solution and the duration of the exposure. A steady state was reached but the initial phase of decrease in P_{Na} was succeeded by a slow continuous rise. This together with the observed persistence of the reduction in P_{Na} after wash out of TX 100 indicated that TX 100 had a partially irreversible action.

Model of TX 100 action

To account for the finding that TX 100 had both a reversible and an irreversible effect on P_{Na} and to provide a basis for quantitative description it was necessary to put up a critical model for the reaction between TX 100 molecules and acceptor sites on the membrane. It was assumed that the reversible effect was due to a reversible binding of TX 100 molecules on acceptor sites on the nodal membrane, thereby blocking the Na per-

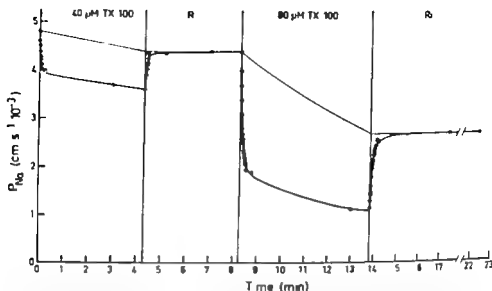
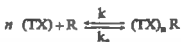


Fig. 2. Time course of TX 100 action on peak P_{Na} . P_N calculated from records of membrane current associated with test pulses to $U=0$ mV repeated 1 per s, and from complete potential clamp runs (Fig. 1). Thin line represents hypothetical time course of irreversible decrease in P_{Na} . Axon 1. Temp. 11–13°C.

meability sites. In a reversible reaction n molecules of TX 100 (TX) react simultaneously with some acceptor (e.g. a receptor) to produce the effect



The ratio $[(\text{TX})_n R]/[R + (\text{TX})_n R]$ is the fractional occupancy due to the reversible reaction of receptor sites which is denoted y_t^{rev} at time t and y_{∞}^{rev} at steady state.

The change in y_t^{rev} during onset and offset should thus satisfy the classical principles of receptor kinetics (Waud 1968). Accordingly the rate constants (k_1 and k_2) were obtained from the experimental time constants for onset and offset and the dissociation constant, $K_D (=k_2/k_1)$, from the steady state curve.

An irreversible decrease in P_N was associated with the exposure to TX 100. The irreversible effect of TX 100 was separated from the reversible changes by measuring P_{Na} at steady state after wash out of the TX 100 solution and interpolating between this P_{Na} value and the value prior to the exposure of TX 100. For the interpolation the irreversible changes were treated as if they followed an exponential time course with a complete irreversible block of P_{Na} at infinite time. The reversible occupancy (y_t^{rev}) was calculated from

$$y_t^{rev} = \frac{P_{Na_0} - P_{Na_t} - \Delta P_{Na_t}^{irrev}}{P_{Na_0} - \Delta P_{Na_{\infty}}^{irrev}} \quad (6)$$

where P_{Na_0} and P_{Na_t} is P_N at beginning and time t of exposure to TX 100 and $\Delta P_{Na_t}^{irrev}$ is the interpolated size of the irreversible decrease in P_{Na} at time t .

Kinetics of TX 100 action on Na permeability

The time course of the effect of various concentrations of TX 100 on the peak P_{Na} was plotted as shown in Fig. 2 and the reversible fractional occupancies (y_t^{rev}) were calculated. The irreversible decrease in P_{Na} was treated as if it followed an exponential time course with a complete irreversible block of P_{Na} at infinite time.

Table I. Reversible effect of TX-100 on P_{Na}

[TX 100] (μ M)	y_{∞}^{rev}	-1	-1.75
		$\frac{1-y_{\infty}^{rev}}{y_{\infty}^{rev}}$ [TX 100] (M 10^{-6})	$\frac{1-y_{\infty}^{rev}}{y_{\infty}^{rev}}$ [TX 100] ^{1.75} (M ^{1.75} 10^{-6})
40	0.19	170	1.42
40	0.12	287	2.44
40	0.30	93	0.77
60	0.60	27	0.24
60	0.71	4	0.29
80	0.38	58	0.78
80	0.66	41	0.56
160	0.71	65	1.44
		96	0.91
		± 91	± 0.72

eq. 2. The thin line drawn in Fig. 2 represents the exponential decrease of P_{Na} due to the reversible reaction. It is evident that, according to the analytical model presently used, the reversible process was at steady state prior to wash out of TX 100 solutions. Therefore y_{∞}^{rev} at the end of the exposure to 40 μ M and 80 μ M TX 100 in this experiment represents y_{∞}^{rev} at equilibrium (y_{∞}^{rev}) at these concentrations of TX-100. Values of y_{∞}^{rev} from this and similar experiments are listed in Table I. The corresponding values of the equilibrium dissociation constant K_D were calculated with $n=1$ and $n=1.75$ respectively. A Hill plot of $y_{\infty}^{rev}/(1-y_{\infty}^{rev})$ vs. [TX 100] had a slope of 1.75 (correlation coeff. = 0.40) meaning that a reaction model with two TX 100 molecules bind to one receptor better describes the present data than a one-reaction. Other, more complex, interpretations will be described below.

The time course for the reversible change in peak P_{Na} during onset of 40 μ M TX-100 and wash offset is plotted in Fig. 3. The reversible fractional occupancy y^{rev} was calculated using eqn. 2 and the time constants were calculated from semilog plots as shown in Figure ($\tau_{on} = 3.7$ s and $\tau_{off} = 4.1$ s). Similar calculations were made of the time constants associated to 80 μ M TX-100 which gave $\tau_{on} = 5.1$ s and $\tau_{off} = 8.1$ s. The rate constants k_1 and k_{-1} were calculated from the time constants. Assuming $n=1.75$ as obtained from the Hill plot (reasonable agreement between k_1 in 40 and 80 μ M was present ($1.37 \cdot 10^6$ and $1.06 \cdot 10^6$ s⁻¹ respectively). Assuming $n=1$ or $n=2$ gave a larger difference between k_1 in the two concentrations. k_1 (and τ_{on}) varied much in these measurements (mean value 0.18 s⁻¹). This is also the case for K_D . K_D calculated as k_{-1}/k_1 using $n=1.75$ and the mean values of k_1 and k_{-1} for 40 and 80 μ M was $1.5 \cdot 10^{-6}$ M^{1.75}. This value is of the same order of magnitude as the K_D value found in the steady-state analysis ($0.91 \cdot 10^{-6}$ M^{1.75}). If the irreversible component of the decrease in P_{Na} is assumed to follow an exponential time course (indicated by the thin line in Fig. 2) it is possible to calculate the time constant τ_{ir} and hence the rate constant (k_{-2}) of this process. In Fig. 4 $1/\tau_{ir}$ versus [TX 100] is plotted for the same fibres as used in the experiments for the steady-state analysis of the reversible reaction. If a first order reaction is

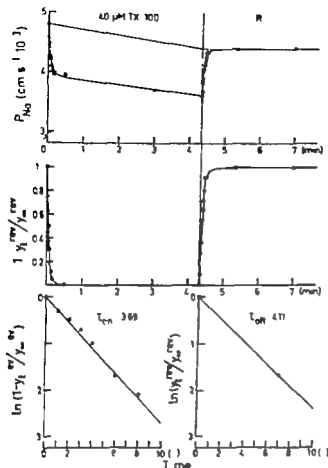


Fig. 3. Time course of change in peak P_{Na} during onset of $40 \mu M$ TX 100 and decay after offset (top). Time constants of reversible action of TX 100 (τ_{on} and τ_{off}) obtained by calculation of reversible occupancy (y^{rev} eqn. 2), calculation of $(1 - y_t^{rev}/y_{\infty}^{rev})$ (middle), and the natural logarithm (plotted on different time scale, bottom). Straight lines (bottom) solutions of eqn. 2 and 3 for indicated values of exponential time constants. Avon 1 Temp. 11 $13^{\circ}C$.

assumed the slope of a regression line fitted to these points should give a value of k_2 according to the eqn. $1/\tau_{ir} = k_2 [TX 100]$. However the points do not fit a straight line well, i.e. the irreversible reaction may deviate from a first order reaction.

Discussion

A large number of chemicals block the specific cation permeability of excitable membranes (cf. Narahashi 1974). Reagents with defined action sites on proteins, e.g. p-chloromercuribenzoic acids (PCMB and PCMBS) and nitrobenzenes block the nervous action potential

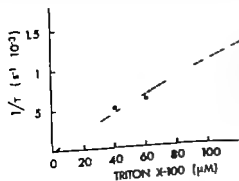


Fig. 4. Dependence of rate of irreversible block of P_{Na} on [TX 100]. Exponential time constant (τ_{ir}) calculated from measurements of irreversible change in P_{Na} after immersion in TX 100 solutions. Necessary assumptions about irreversible reaction described in text. Rate constant (k_2) defined by eqn. $1/\tau_{ir} = k_2 [TX 100]$ was $11.0 \pm 3.0 (s^{-1} M^{-1})$. Interrupted line solution of this eqn. for $k_2 = 11.0$. Temp. 11 $13^{\circ}C$.

through a suppression of P_{Na} and P (Smith 1958, Cooke *et al.* 1968). Several of these compounds have an irreversible action.

The present investigation shows that application of TX 100 decreases P_{Na} in the nodal membrane of frog myelinated nerve fibres. When solutions of low concentrations of TX 100 ($10 \mu M$ and $20 \mu M$) were applied for short times the decrease in P_{Na} was to its main part reversible at wash out of the TX 100 solution. A large irreversible change on the Na permeability mechanism appeared at high [TX 100] or when long exposure times were tried. At such conditions there was finally a rapid increase in membrane leak conductance. The present findings are in agreement with earlier observations that nonionic detergents block the ionic conduction in nerve and the neuromuscular transmission (see Introduction). It has earlier been shown that TX 100 affects excitability in frog muscle fibres, thus Rydqvist (1971) found that it decreased the maximum rate of rise of the conducted action potential.

With the potential clamp technique used in the present experiments it was possible to monitor the time course of TX 100 action on the nodal membrane by recording the membrane currents associated with repeated potential pulses of a constant amplitude. This, in combination with the short time for solution change (half time 100–150 ms), gave a satisfactory resolution for an analysis of the kinetics of TX-100 action. The analysis was, however, complicated by the irreversible decrease in P_{Na} which accompanied the application of TX-100 solutions. The size of the irreversible component of TX-100 action during onset could not be directly measured, but was appreciated by interpolation between P_{Na} prior to the application of the test solution and P_{Na} at steady state after washout. The analysis of the reversible reaction strongly suggested a deviation from a first order reaction between TX-100 molecules and membrane receptors. From the Hill plot n was estimated to be 1.75 which gave a dissociation constant K_D $0.91 \cdot 10^{-6} M^{1.75}$. Similarly Rydqvist (1977) found that $n = 1.57$ for the measurements of TX 100 action on the maximum rate of rise of the action potential in frog muscle. In general, many pharmacological effects on excitable membranes do not follow first order reactions (Rang 1971) and often give Hill coefficients greater than one. If n is an integer this can be interpreted as described in eqn. (1) i.e. n ligands react simultaneously with a receptor to give the effect. An alternative interpretation is that the measured effect, in this case the change in P_{Na} , cannot be translated to the formation of the reaction product. It is for example possible that several receptors are connected to one Na channel. This assumption is not entirely hypothetical since there are reasons to believe that the opening of a Na channel is dependent on the cooperation of several (in squid axons three) gating molecules (Hodgkin and Huxley 1952, Keynes and Rojas 1974) which might correspond to the reaction sites of the TX 100 molecules. The finding that n is a noninteger could be accounted for by assuming an allosteric effect, i.e. the affinity of sites are mutually influenced by the occupation of other sites (Monod, Wyman and Changeux 1965).

Nonionic detergents can interact with the lipid bilayer phospholipids and hydrophobic proteins. It is likely that the permeability blocking action is due to a reaction with membrane proteins associated to the permeability mechanism. TX 100 has been used for the solvation of the acetylcholine receptor glycoprotein from different electrical organs (Miledi and Potter 1971, Mossier, Olsen and Changeux 1972). The irreversible decrease in P_{Na} was considerably slower than the reversible effect of TX-100, and an increase in leak conductance appeared

when a large part of the Na permeability mechanism had been irreversibly damaged. The mechanism for the irreversible effects might similarly be a solvation of the proteins through the lipid-detergent interaction from the permeability sites of the nodal membrane, thus causing a permanent decrease in P_{Na} succeeded by an increase in the leak conductance.

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Dose-dependent inhibition of sensory nerve activity in the feline dental pulp by anti-inflammatory drugs

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Abstract

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The aim of the present study was to investigate whether substances known to antagonize algogenic agents can block an experimentally induced increased sensory nerve activity in the dental pulp of the cat. Increased neural activity following repeated brief heat stimulation has been shown to be characteristic of the individual cat and can be utilized for intrasubject comparative studies on such increased activity. When indomethacin, diclofenac sodium or naproxen were given intravenously as pretreatment prior to stimulation, a consistent blocking effect on expected impulse activity as compared to the impulse activity in the control teeth was produced. Indomethacin produced dose-dependent inhibition (ID_{50} 0.04-0.2 mg/kg). Diclofenac sodium (5 mg/kg) inhibited nerve activity by 31-91% and naproxen (15 mg/kg) by 92-95%. Methysergide (0.02-0.08 mg/kg) did not show such blocking effect. When the drugs were administered during state of established increased neural activity no effect on the current impulse activity was obtained. The present results suggest that prostaglandins may be involved in certain stages of heat induced pulp inflammation and act as mediators of increased intradental sensory nerve excitability.

Pain produced by inflammatory processes is considered to be due to release of endogenous algogenic agents which then excite the nerve endings or increase their susceptibility to stimulation. Keele and Armstrong (1964-1968) suggested that the increased receptor excitability is the most important factor in causing inflammatory pain. According to Sicurti (1968) increased sensitivity may be produced by 5-hydroxytryptamin and Olgart (1974) showed that this substance excites intradental nerves when applied to a dentinal cavity. Other substances which have been found in inflamed tissue and may be involved in the increase in the excitability are prostaglandins and their unstable intermediates (Vane 1972, Ferreira 1973). However findings by Haegerstrom and Edwall (1977) seemed to exclude a role of prostaglandins as mediators of pain in the dental pulp.

Recent investigations have shown that pathological alterations including increased sensory nerve activity can be induced in the dental pulp by means of repeated brief heat stimulation of the tooth surface (Ahlberg 1978). This method to induce and record an increased intradental neural activity provides a reproducible experimental model to study which algogenic

24 again are involved in producing such increased activity during inflammatory processes in the pulp.

The aim of the present study was to investigate whether non-steroid anti-inflammatory drugs, which interfere with prostaglandin synthesis and the serotonin blocking drug methysergide could block an experimental, heat-induced increase in sensory nerve activity in the distal pulp of the cat. Among the anti-inflammatory drugs indomethacin, diclofenac sodium and d-nitrophen were selected for this study. These drugs have previously been shown to inhibit prostaglandin synthesis (Tomlinson *et al.* 1972, Vane 1972, Ku *et al.* 1975) and recent studies on renal prostaglandins indicate that diclofenac sodium might be used as an alternative to indomethacin (Oluf Lundin and Ånggård, in press 1978).

Material and Methods

Experiments were carried out on adult cats (2-3 kg) anaesthetized with chloralose (40 mg/kg i.v.) or sodium (30 mg/kg) or in some cases with sodium pentobarbital (30 mg/kg). Immobilization and surgery preparations were performed as described earlier by Olsson, Hagerstrand and Nil (1974). Electrical potentials from intradental sensory nerves were recorded from cavities extending at half the distance from crown to pulp. The floor of the cavities are covered with a layer of amalgam which insulates steel tubes were secured by composite filling material as described by Edvall and Olsson (1977). Recording cables are connected to the tubes and conventional techniques were used to display the signals on oscilloscope tape. The signals were counted on a scaler and printed out for 10 min and impulse frequency was given as impulses/s. A thermometer connected to a stainless steel tube in the tooth surface afforded simultaneous registration of changes in the temperature

of the tooth surface by application for approximately 1 min of heated gutta-percha at about 70°C, provokes an immediate increase in recorded neural activity which generally lasts 10-20 s. This first response may be regarded as a physical response to stimulation. When heat stimulations are repeated subsequent more prolonged increases in nerve activity (phase 3) occurs after a latent interval (phase 2) following the phase 1 burst of impulses (Fig. 1A).

The study concerned with phase 3 as defined above. A quantitative expression of this activity was used by calculating the mean number of impulses per second over the period from 1-4 min inclusive in stimulation 1 excluding the first and second phases of the response. This parameter was reproducible in individual cats for stimulation of different enamel teeth prepared at the same time, variation being as within homogeneous pairs of teeth (Åkberg 1978). The experimental procedure was carried out in the following way. After preparation each enamel tooth was stimulated with cold by applying cotton pellet soaked in ethyl chloride to the tooth surface. This stimulus has been shown to elicit bursts of impulses (Jägerskiöld 1970) and was used as a test of the responsiveness to stimulation. The controls, generally one upper and one lower canine tooth were selected by random choice. The control teeth were stimulated possibly with heat as described above until the maximum impulse activity was obtained. After intradental injection of the drug to be tested, the procedure was repeated on the test teeth. Maximum phase 3 activity was calculated for tests and controls and blocking effects of the drugs were expressed as per cent blocked.

In another series of experiments the blocking substance was given during a state of established heat induced neural activity instead of as pretreatment.

The following drugs were tested.

Indomethacin, 1-(α -chlorobenzoyl)-5-methoxy-2-methylindole-3-carboxylic acid 0.04-0.2; 1-5 mg/kg b.w. in 0.1 mol sodium phosphate buffer pH 8.

Diclofenac sodium, 0-(2,6-dichlorophenyl)-amino)-phenylacetic acid sodium 5 mg/kg b.w. in 5-7 ml isotonic saline solution.

d-Nitrophen, 4-X6-methoxy-2-amphyl-propionic acid 15 mg/kg b.w. in 9 ml 0.1 mol sodium phosphate buffer pH 8.

Methysergide, 9,10-didehydro-N(1-(hydroxy-methyl)propyl)-1,6-dimethylergoline-8-carboxamide 0.02-100 mg/kg b.w. in 1-5 ml isotonic saline solution.

Unless specifically stated, drugs given as pretreatment were administered 30 to 60 min before stimulation. The rate of injection was about 1 ml in 30 s.

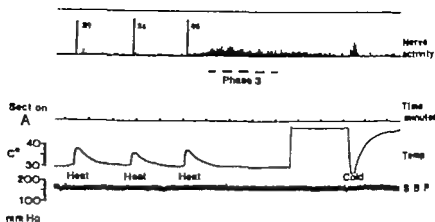


Fig. 1a

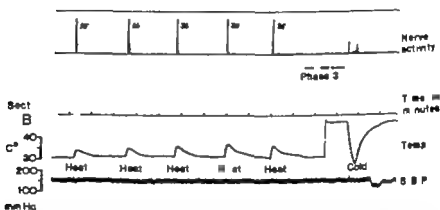


Fig. 1b

Fig. 1 Blocking effect by naproxen 15 mg/kg i.v. on expected heat induced impulse activity. Section A shows recordings from the control tooth. In section B recordings from the test tooth are shown 25 min after drug administration. Records are from above, intradental nerve activity, time, temperature and systemic blood pressure. Temperature is registered at the tooth surface at 1/2 crown length. Note the shift in base line between the last heat and the cold stimulation. Temperature calibration refers to the heat stimulations. The minimum temperature recorded during cold stimulation was about 10°C. Mean impulse frequency for period marked --- was in section A 17.6 imp/s and in section B 1.5 imp/s calculated from the scaler output.

Results

Blocking agents injected i.v. as pretreatment effect on expected heat induced activity

Indomethacin, diclofenac sodium and naproxen were found to be potent inhibitors of expected increased sensory nerve activity. Fig. 1 shows a typical protocol. An immediate response to heat stimulation occurred in the control tooth (Fig. 1 section A) and the first and second stimulations caused only a low phase 3 activity. The third stimulation, however, elicited marked impulse activity after a delay of about 1 min. The average number of impulses per second during this maximum phase 3 activity was 17.6. After about 6 min when the activity had largely subsided, cold stimulation caused a clearcut increase in impulse frequency. In section B recordings from the test tooth are shown 25 min after naproxen 15 mg/kg i.v. The immediate response to heat was the same as in the control tooth but the maximum phase 3 activity reached only 1.5 impulses per second despite repeated heat

Table 1. Effect of pretreatment with drugs on phase 3 activity

Drug	Control		Pretreatment		Reduction per cent of control $\bar{X} \pm S.D.$
	Number of teeth	Mean phase 3 activity Imp/sec $\bar{X} \pm S.D.$	Number of teeth	Mean phase 3 activity Imp/sec $\bar{X} \pm S.D.$	
Indomethacin 5 mg/kg	5	15.7 \pm 4.6	7	2.0 \pm 0.9	86 \pm 7.9 $p < 0.001$
Diclofenac sodium 5 mg/kg	9	7.7 \pm 2.9	13	2.0 \pm 1.6	75 \pm 12.3 $p < 0.001$
Naproxen 15 mg/kg	2	19.2 \pm 2.3	2	1.3 \pm 0.3	93 \pm 2.1

Student's *t* test.

stimulation. Cold stimulation at the end of the section can be regarded as a test of the responsiveness of the tooth. No obvious changes in blood pressure were recorded following injection of the drug. The blocking effect as demonstrated here was a consistent finding in all experiments using indomethacin, diclofenac sodium and naproxen and the magnitudes of the reduction are shown in Table 1.

The relationship of dose and the blocking effect of indomethacin was studied in 11 cats, 19 teeth. Doses from 0.04 to 5 mg/kg were administered, generally only one dose being given in each animal and the effects observed in one or two test teeth (3 and 7 cats respectively). In one cat two doses were given. The results are shown in Fig. 2. Blockade of neural activity was seen at the lowest dose studied (0.04 mg/kg) and ranged from zero to 36 per cent with a mean value of 15% (S.D. $\approx \pm 17.5$) of control. When the dose was increased to 0.2 mg/kg the blocking effect ranged from 72 to 88 per cent with a mean value of 81% (S.D. ± 7.0). A dose of 1 mg/kg resulted in a blocking effect of 91 per cent which was not increased by raising the dose to 5 mg/kg. In the cat given two doses the first dose (0.04 mg/kg) resulted

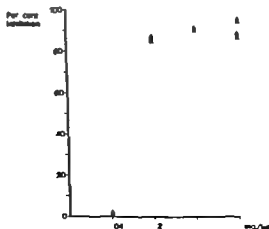


Fig. 2. Blocking effect by indomethacin 0.04-5 mg/kg i.v. on expected heat induced impulse activity inhibition expressed in per cent of control is marked on the ordinate.

in a blocking effect of 8.3 per cent. When the dose was increased to 0.2 mg/kg the blocking effect rose to 85 per cent as compared to control. Data from this expt. are included in Fig. 2.

In similar expts. pretreatment with methysergide injected 3, 30, 60 or 90 min before stimulation was ineffective in blocking the expected activity (5 teeth, 3 cats).

Blocking agents injected i.v. during established heat induced activity

In 15 cats the test drugs were given after the establishment of clearcut phase 3 activity. Alteration in this activity was seen with indomethacin (4 teeth), diclofenac sodium (4 teeth), naproxen (1 tooth), methysergide (8 teeth).

Discussion

The present study shows that indomethacin, diclofenac sodium and naproxen administered as a pretreatment could inhibit an expected, heat induced increased activity in the rat dental sensory nerve. Methysergide did not show such blocking effect.

Indomethacin, diclofenac sodium and naproxen are characterized by their analgesic and anti-inflammatory properties (Flower 1974). It has been suggested that these effects are due to blockade of the prostaglandin synthesis (Vane 1972) and that the specific inhibitory action of the aspirin-like drugs is directed against the prostaglandin synthetase enzyme cyclooxygenase (Bergström 1977). Since this enzyme is found in most mammalian tissues it seems probable that it is present in the dental pulp also. The blocking effect of indomethacin, diclofenac sodium and naproxen on the heat induced sensory nerve activity seems to indicate that prostaglandins and/or their intermediates are involved in producing this increased neural activity. It must be borne in mind, however, that these compounds also exert inhibitory effects on other enzymes and cellular systems. The present finding that the blocking effect of indomethacin was dose dependent in the range 0.04–1 mg/kg supports the assumption that the effect was due to inhibition of prostaglandin biosynthesis since few of these other effects would be expected to occur at the low doses which were found in this study to inhibit sensory nerve activity (cf Flower 1974).

Methysergide blocks some 5-hydroxytryptamin receptors (Gaddum and Hameed 1954) but there is no evidence that it affects prostaglandin synthesis (cf Flower 1974). The finding that methysergide in pharmacological doses, which have been shown to block neural activity induced by compound 48/80 (Gazellus and Olgart 1977, unpublished data), had no inhibiting effect on expected, heat induced sensory nerve activity indicates that 5-hydroxytryptamin is not involved in producing such increase in intradental activity.

The possible participation of prostaglandins in causation of increased intradental nerve activity has been studied by Haegerstad and Edwall (1977) using various ways to induce neural activity. They found that activity induced by compound 48/80 applied to open cavities could not be blocked by indomethacin (5 mg/kg) or sodium acetylsalicylate (200 mg/kg) given i.v. or (2.5 mg/ml and 9 mg/ml respectively) applied locally. However, the inhibitors of prostaglandin synthesis were given after the increased nerve impulse activity was established and this might in part explain their negative findings. They also found that immersion of the tooth surface to 45–50°C in combination with close i.e.

Injection of prostaglandins or the PG-precursor arachidonic acid did not produce any neural activity. It seems likely that the heat stimulus was inadequate to produce inflammatory fluid changes in the pulp.

Neither the immediate physical response to heat stimulation nor the response to cold were affected by pretreatment with indomethacin, diclofenac sodium or naproxen. These findings indicate that these drugs do not influence the excitability of the sensory nerves *per se* and furthermore, implies that prostaglandins are not essential to neural activity produced in normal, uninfamed tissue. This latter suggestion is supported by the finding reported by Hagerstrom and Edwall (1977) that prostaglandin PGE₂ did not induce any nerve impulses when applied to a dentinal cavity.

The drugs, which were effective as pretreatment, were unable to influence a state of established increased activity. This may be a consequence of local ischemia at the site of the thermal injury preventing the drug from reaching the area where algogenic agents are present. This hypothesis is supported by recent findings that neural activity induced by a depot of compound 48/80 applied to a dentinal cavity could not be abolished by injection of tetrodotoxin or methysergide, whereas pretreatment with these drugs inhibited the activity (Garellis and Olqart 1977 unpublished data). Since compound 48/80 probably causes reduced blood flow in the vicinity of the depot (Ahlberg and Edwall 1977) the lack of effect of the blocking agent might be due to incomplete distribution of the agent to the pulp under the cavity.

In conclusion, the results of the present study indicate that prostaglandins are involved in certain stages of heat induced pulp inflammation and that they in this situation act as mediators of increased activity in the sensory nerves of the pulp.

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in a blocking effect of 8.3 per cent. When the dose was increased to 0.2 mg/kg the blocking effect rose to 85 per cent as compared to control. Data from this expt. are included in Fig. 2.

In similar expts. pretreatment with methysergide injected 3, 30, 60 or 90 min before stimulation was ineffective in blocking the expected activity (5 teeth, 3 cats).

Blocking agents injected i.v. during established heat induced activity

In 15 cats the test drugs were given after the establishment of clearcut phase 3 activity. No alteration in this activity was seen with indomethacin (4 teeth), diclofenac sodium (4 teeth), naproxen (1 tooth), methysergide (8 teeth).

Discussion

The present study shows that indomethacin, diclofenac sodium and naproxen administered i.v. as a pretreatment could inhibit an expected, heat induced increased activity in the intradental sensory nerves. Methysergide did not show such blocking effect.

Indomethacin, diclofenac sodium and naproxen are characterized by their analgesic and anti-inflammatory properties (Flower 1974). It has been suggested that these effects are due to blockade of the prostaglandin synthesis (Vane 1972) and that the specific inhibitory action of the aspirin-like drugs is directed against the prostaglandin synthesizing enzyme cyclooxygenase (Bergström 1977). Since this enzyme is found in most mammalian tissues it seems probable that it is present in the dental pulp also. The blocking effect of indomethacin, diclofenac sodium and naproxen on the heat induced sensory nerve activity seems to indicate that prostaglandins and/or their intermediates are involved in producing this increased neural activity. It must be borne in mind, however, that these compounds also exert inhibitory effects on other enzymes and cellular systems. The present finding that the blocking effect of indomethacin was dose dependent in the range 0.04–1 mg/kg supports the assumption that the effect was due to inhibition of prostaglandin biosynthesis since few of these other effects would be expected to occur at the low doses which were found in this study to inhibit sensory nerve activity (cf Flower 1974).

Methysergide blocks some 5-hydroxytryptamin receptors (Gaddum and Hameed 1954) but there is no evidence that it affects prostaglandin synthesis (cf Flower 1974). The finding that methysergide in pharmacological doses, which have been shown to block neural activity induced by compound 48/80 (Gazellius and Olgart 1977, unpublished data), had no inhibiting effect on expected, heat induced sensory nerve activity indicates that 5-hydroxytryptamin is not involved in producing such increase in intradental activity.

The possible participation of prostaglandins in causation of increased intradental nerve activity has been studied by Hägerström and Edwall (1977) using various ways to induce neural activity. They found that activity induced by compound 48/80 applied to open cavities could not be blocked by indomethacin (5 mg/kg) or sodium acetylsalicylate (200 mg/kg) given i.v. or (2.5 mg/ml and 9 mg/ml respectively) applied locally. However, the inhibitors of prostaglandin synthesis were given after the increased nerve impulse activity was established and this might in part explain their negative findings. They also found that increase of the temperature at the tooth surface to 45–50°C in combination with close la.

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Sympathetic control of metabolic and hormonal responses to exercise in rats

By

H. GALBO, E. A. RICHTER, N. J. CHRISTENSEN and J. J. HOLST

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Abstract

GALBO, H., E. A. RICHTER, N. J. CHRISTENSEN and J. J. HOLST. Sympathetic control of metabolic and hormonal responses to exercise in rats. *Acta physiol. scand.* 1978, 102, 441-449

The importance of the sympatho-adrenal system for the pancreatic hormonal responses to exercise and, furthermore, the role of glucagon and catecholamines for the hepatic glycogen depletion during exercise was tested. Rats were either surgically adrenalectomized and chemically sympathectomized, left or sham-operated. Two weeks later the rats had either rabbit-antiglucagon serum or 6-hydroxydopamine or sham-operated. Subsequently the rats either rested or swam with tail weight for 75 min. Arterial blood serum injected. Subsequently the rats either rested or swam with tail weight for 75 min. Immediately after and during exercise blood was drawn and liver and muscle tissue collected. In control rats there was an increase in blood glucose concentrations during exercise plasma insulin concentrations were unchanged, but glycogen concentrations increased. In sympathectomized rats, compared to control rats, glycogen concentrations increased less, and insulin concentrations were higher although glucose concentrations were lower during exercise. Sympathectomy completely abolished the exercise-induced decrease in liver and muscle glycogen concentrations, whereas neither glycogen depletion nor plasma catecholamine concentrations were affected by the administration of glucagon antibodies. These findings indicate that the sympatho-adrenal system enhances glucagon secretion as well as muscular and hepatic glycogen depletion but inhibits insulin secretion in exercising rats. The increase in glucose concentrations, however, does not enhance hepatic glycogen depletion at the work load used.

Key words: Exercise, epinephrine, norepinephrine, glucagon levels, sympathetic nervous system, liver, muscle, glycogen, sympathectomy, adrenal medulla.

During prolonged exercise in man as well as in the rat the secretion of glucagon increases, whereas the secretion of insulin decreases. In both species the decrease in insulin secretion during exercise has been ascribed to a catecholamine mediated inhibition of pancreatic β -cell secretion (Galbo, Christensen and Holst 1977 c, Brisson, Malaisse-Lagae and Malaisse 1977). As to the regulation of glucagon secretion, however, in these two species different results have been reported to be of major importance during exercise. In man the exercise induced increase in glucagon secretion could not be reduced by adrenergic blocking drugs (Galbo et al. 1977 a, 1977 c) but was markedly reduced by maintenance of euglycaemia by

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The importance of the sympathetic-adrenal system for the pancreatic hormonal response to exercise and, likewise, the role of glucagon and catecholamines for the hepatic glycogen depletion during exercise was studied. Rats were either surgically adrenalectomized and chemically sympathectomized with 6-hydroxydopamine or sham-treated. Two weeks later the rats had either rabbit-anti-glucagon serum or normal rabbit serum injected. Subsequently the rats either rested or swam with tail weight for 75 min. Immediately afterwards cardiac blood was drawn and liver and muscle tissue collected. In control rats in spite of an increase in blood glucose concentrations during exercise plasma insulin concentrations were unchanged, while glucagon concentrations increased. In sympathectomized rats, compared to control rats, glucagon concentrations increased less, and insulin concentrations were higher, although glucose concentrations were lower during exercise. Sympathectomy completely abolished the exercise-induced decrease in liver and muscle glycogen concentrations, whereas neither glycogen depletion nor plasma catecholamine concentrations were influenced by the administration of glucagon antibodies. These findings indicate that the sympathetic-adrenal system enhances glucagon secretion as well as muscular and hepatic glycogen depletion but inhibits insulin secretion in exercising rats. The increase in glucagon concentrations, however, does not enhance hepatic glycogen depletion at the work load used.

Key words. Exercise, epinephrine, norepinephrine, glucagon insulin, sympathetic nervous system, liver, muscle, glycogen, sympathetic adrenal medulla

During prolonged exercise in man as well as in the rat the secretion of glucagon increases, whereas the secretion of insulin decreases. In both species the decrease in insulin secretion during exercise has been ascribed to a catecholamine mediated inhibition of pancreatic β -cell secretion (Galbo, Christensen and Holst 1977 c, Brinson, Malaisse-Lagae and Malaisse 1971). As to the regulation of glucagon secretion, however, in these two species different results have been reported to be of major importance during exercise. In man the exercise-induced increase in glucagon secretion could not be reduced by adrenergic blocking drugs (Galbo *et al.* 1977 a, 1977 c) but was markedly reduced by maintenance of eudromia by

glucose infusion during exercise (Galbo *et al.* 1977a). These studies indicate that in man glucose availability is the major determinant of pancreatic A-cell secretion during exercise. From studies of immunosympathectomized rats (Luyckx *et al.* 1975) and of rats treated with adrenergic blocking drugs (Luyckx and Lefebvre 1974) it has been concluded that in the rat catecholamines play a major role in the exercise-induced glucagon secretion. Recently however it has been shown that the glucagon as well as the catecholamine response to exercise are diminished in trained rats, which maintain high blood glucose concentration for a longer period of time during exercise than untrained rats (Galbo *et al.* 1977b). These findings are compatible with both the concept that glucose and the concept that catecholamines play the major role in exercise induced glucagon secretion.

It has been shown that the decrease in hepatic glycogen content elicited during prolonged heavy exercise in rats can be reduced by administration of glucagon-antibodies (Galbo and Holst 1976). This finding suggests that glucagon enhances hepatic glycogen depletion during exercise. However, also in antibody treated exercising rats a decrease in hepatic glycogen concentrations was observed. Accordingly it was concluded that other stimuli than glucagon possibly catecholamines, may enhance hepatic glycogen depletion during exercise.

In the present study of rats, which were chemically sympathectomized and surgically adrenalectomized, we have tried further to evaluate the significance of the sympathetic-adrenal system for the pancreatic hormonal secretion during exercise. Furthermore, by administration of glucagon-antibodies to sympathectomized as well as to control rats we tried to evaluate the relative role of glucagon and catecholamines for the hepatic glycogen depletion during exercise.

Material and methods

69 male Wistar rats weighing 230–250 g were anesthetized with ether and either adrenalectomized or sham operated. Adrenalectomy was performed by electrocoagulation through bilateral lumbar incisions. The first week after the operation the rats had free access to 0.9% NaCl solution in water. In order to allow recovery of cortical tissue the surgery was carried out 15 days prior to the exercise test.

In adrenalectomized rats chemical sympathectomy was accomplished according to the procedure, which has been found to result in the most complete destruction of adrenergic nerve terminals in rats (Thoenen and Tranzer 1968). Two days after surgery the animals received (in a tail vein and six hours apart) two injections of 34 mg/kg b.wt.⁻¹ 6-hydroxydopamine dissolved immediately before injection in 0.5 ml 0.9% NaCl containing 0.5 mg ascorbic acid. One week later the rats received further 2 × 68 mg/kg⁻¹ 6-hydroxydopamine. Sham operated rats had only the solvent injected.

The exercise test was carried out in the morning, and the rats fasted 12 h in advance. During light ether anesthesia 0.3 ml of blood were drawn by cardiac puncture for glucose analysis and 0.5 ml of either rabbit antyglucagon serum or normal rabbit serum were injected through the cardiac cannula. After 15 min of recovery the rats were either forced to swim with a tail weight of lead (2% of b.wt.) in water maintained at 33–34°C for 75 min, or they remained resting. The number of rats assigned to the various procedures appear from the figures and tables. At the end of exercise or equivalent period of rest, the rats were superficially anesthetized with ether and 8 ml of blood were drawn by cardiac puncture. Then samples of the liver of the superficial part of the vastus lateralis muscle (which consists predominantly of fast-twitch white fibers), of the deep part of the vastus lateralis muscle (predominantly fast-twitch red fibers), of the soleus muscle (predominantly slow-twitch red fibers) and of the pancreas were quickly removed and frozen in liquid nitrogen. In sympathectomized rats the integrity of the adrenal cortex and the absence of chromaffin cells were verified by light microscopy of two sections, stained by hematoxylin and eosin, of each adrenal gland.

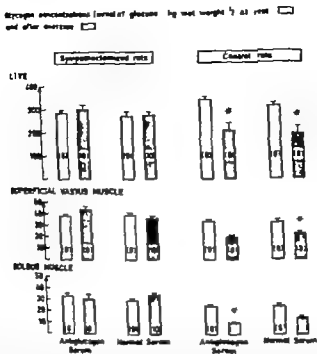


Fig. 1. The effect of 75 min of forced swimming on the concentrations of glycogen in the liver and in two muscles with different fiber types. Sympathetomized rats were adrenalectomized and treated with dehydroepiandrosterone two weeks before sacrifice. Antagonagon serum or normal rabbit serum was injected immediately prior to exercise or equivalent period of rest. Values are mean \pm S.E. Number of observations is shown in parentheses. Asterisk signifies $P < 0.05$ for difference between values at rest and after exercise. In all muscular glycogen concentrations are significantly higher ($p < 0.05$) in sympathetomized than in control rats.

The methods of analysis of blood and tissue for hormones and metabolites have been reported elsewhere (Gallo and Holt 1974, Gallo *et al.* 1977 b). All analyses were performed at least in duplicate and all radiochemical determinations of hormones were carried out in single assay run. Tissue samples were stored at -80°C and analysed in random order. Glycogen concentrations were expressed as nmol of glucose/kg wet tissue $^{-1}$. The precision of the glycogen determinations calculated as the coefficient of variation was 7% at 36 nmol/kg $^{-1}$ for double determinations of nine samples (4–14 μg) of the same track, and 4% at 306 nmol/kg $^{-1}$ for double determinations on 6 samples from the same liver. The characteristics of the antagonagon serum that was administered to the rats have been described in detail elsewhere (Gallo and Rachee 1973). At sacrifice 300 μl of 1:1 400 diluted plasma from rats treated with antagonagon serum were able to bind 40% of 113 fmol 125-I-labelled glucagon. Glucose concentrations were assessed by the t-test for paired comparisons, and catecholamine concentrations by the t-test for unpaired comparisons (Snedecor and Cochran 1965). The remaining data were subjected to analysis of variance preceded by Bartlett's test, and significant differences were located by Tukey's test (Snedecor and Gallo 1974).

Results

In control rats treated with normal serum glycogen concentrations in liver as well as in skeletal muscle had significantly decreased after 75 min of swimming (Fig. 1 and Table II). Simultaneously the concentrations in plasma of glucagon, glucose, lactate and pyruvate

glucose infusion during exercise (Galbo *et al.* 1977a). These studies indicate that in glucose availability is the major determinant of pancreatic A-cell secretion during exercise. From studies of immunosympathectomized rats (Luyckx *et al.* 1975) and of rats treated with adrenergic blocking drugs (Luyckx and Lefebvre 1974) it has been concluded that in the rat catecholamines play a major role in the exercise-induced glucagon secretion. Recently, however, it has been shown that the glucagon as well as the catecholamine response to exercise are diminished in trained rats, which maintain high blood glucose concentrations for a longer period of time during exercise than untrained rats (Galbo *et al.* 1977b). These findings are compatible with both the concept that glucose and the concept that catecholamines play the major role in exercise-induced glucagon secretion.

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Table 1. Tissue catecholamine concentrations ($\mu\text{g}\cdot\text{g}^{-1}$ wet weight) in sympathectomized (SX) and control rats (C).

	Pancreas		Liver		Deep ventral muscle	
	SX	C	SX	C	SX	C
Norepinephrine	32.3 ± 7.3^a (7)	239.0 ± 27.0 (7)	1.1 ± 0.4 (8)	24.2 ± 2.5 (8)	21.8 ± 2.5 (9)	81.2 ± 6.0 (8)
Epinephrine	0.6 ± 0.5 (8)	14.7 ± 2.1 (7)	0.6 ± 0.6 (7)	0.2 ± 0.2 (7)	4.5 ± 1.1^b (8)	1.0 ± 0.4 (8)

Values are mean \pm S.E. Number of observations are shown in parentheses. Samples of the pancreas were obtained from exercised rats, whereas liver and muscle samples were obtained from resting rats. ($p < 0.001$) and ($p < 0.05$) denote that SX rats differ significantly from C rats.

$p < 0.05$, $n = 7$) but not in sympathectomized rats. In resting rats sympathectomy significantly increased the concentrations of muscle glycogen (Fig. 1 and Table III) and significantly decreased the concentration of glucagon in plasma (Fig. 2). During exercise neither the concentrations of glycogen in liver¹ and muscle (Fig. 1 and Table III) nor the concentration of glucose in blood (Fig. 2) changed in sympathectomized rats. Furthermore, during exercise the concentration of glucagon in plasma increased less in these rats than in control rats (Fig. 2). The concentration of insulin in plasma was higher in exercised sympathectomized rats than in both exercised and resting control rats (Fig. 2). The responses of the remaining variables to exercise were not influenced by sympathectomy.

Effect of antiglucagon serum

In resting rats the concentration of alanine in plasma was increased by the administration of antiglucagon serum in control as well as in sympathectomized rats (Table III). Apart from this, comparisons of rats treated with antiglucagon serum with rats treated with normal serum did not reveal any consistent differences. Thus, the plasma concentrations of catecholamines were not altered by antiglucagon serum (Table II).

Table 2. Plasma concentrations ($\mu\text{g}\cdot\text{ml}^{-1}$) of norepinephrine and epinephrine in rats after 10 min of swimming.

	Sympathectomized and glucagon-depleted	Glucagon-depleted	Controls
Norepinephrine	0.92 ± 0.09^a (7)	3.48 ± 0.84 (7)	3.14 ± 0.39 (7)
Epinephrine	0.36 ± 0.12^a (7)	1.52 ± 0.39 (7)	1.40 ± 0.32 (7)

Values are mean \pm S.E. Number of observations are shown in parentheses. Glucagon-depleted rats had been treated with antiglucagon serum and controls with normal rabbit serum. ^a denotes that the sympathectomized group of rats differed significantly ($p < 0.02$) from the other groups.

¹The lack of decrease of hepatic glycogen concentrations was verified by reanalysis of all liver samples.

The concentrations of glucose (mmol l⁻¹) in blood and of insulin (pmol l⁻¹) and glucagon (pmol l⁻¹) in serum at rest (□) and after exercise (▨).

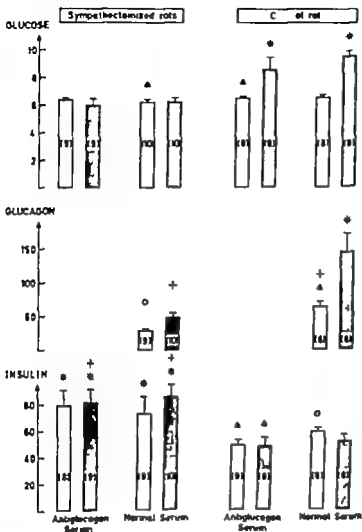


Fig. 2. Values are mean \pm S.E. Number of observations are shown in parentheses. Values are significantly higher than Δ -values of the same variable ($p < 0.05$), and \dagger -values are significantly higher than Δ -values ($p < 0.05$). Blood for glucose analysis was obtained from each rat before as well as after exercise, whereas blood for hormone analyses was obtained only after exercise or equivalent period of rest. For further explanation see Fig. 1.

had increased (Fig. 2 and Table III), whereas the plasma concentrations of FFA, glycerol, alanine and insulin did not change significantly. During exercise in control rats glucagon concentrations correlated significantly with norepinephrine ($r = 0.91$, $p < 0.01$, $n = 7$) as well as with epinephrine ($r = 0.77$, $p < 0.05$, $n = 7$) concentrations in plasma. In none of the resting groups of rats did the blood glucose concentration change significantly during the 90 min rest period (not shown).

Effects of sympathectomy

The concentrations of catecholamines in pancreas, liver and skeletal muscle (Table I) as well as in plasma (Table II) were significantly diminished by the applied combination of chemical sympathectomy and adrenalectomy. The concentrations of epinephrine and norepinephrine in plasma correlated significantly during exercise in control rats ($r = 0.87$

TABLE I. Tissue catecholamine concentrations ($\mu\text{g}\cdot\text{g}^{-1}$ wet weight) in sympathectomized (EX) and control rats (C)

	Pancreas		Liver		Deep vastus muscle	
	EX	C	EX	■	EX	C
Norepinephrine	32.3 ± 7.5^a (7)	259.0 ± 27.0 (7)	1.1 ± 0.4 (8)	24.2 ± 2.5 (8)	21.8 ± 2.5 (8)	81.2 ± 6.0 (8)
Epinephrine	0.6 ± 0.5 (7)	14.7 ± 2.1 (7)	0.6 ± 0.6 (7)	0.2 ± 0.2 (7)	4.5 ± 1.1^b (8)	1.8 ± 0.4 (8)

Values are mean \pm S.E. Number of observations are shown in parentheses. Samples of the pancreas were taken from exercised rats, whereas liver and muscle samples were obtained from resting rats. ($p < 0.001$) and ($p = 0.07$) denote that EX rats differ significantly from C rats.

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Effects of antidiabetic serum

In resting rats the concentration of alanine in plasma was increased by the administration of antidiabetic serum in control as well as in sympathectomized rats (Table III). Apart from this, comparisons of rats treated with antidiabetic serum with rats treated with normal serum did not reveal any consistent differences. Thus, the plasma concentrations of catecholamines were not altered by antidiabetic serum (Table II).

TABLE II. Plasma concentrations ($\mu\text{g}\cdot\text{ml}^{-1}$) of norepinephrine and epinephrine in rats after 75 min of swimming.

	Sympathectomized and glucagon-depleted	Glucagon-depleted	Controls
Norepinephrine	0.92 ± 0.09^a (7)	1.48 ± 0.84 (7)	2.14 ± 0.59 (7)
Epinephrine	0.36 ± 0.12^a (7)	1.52 ± 0.39 (7)	1.40 ± 0.12 (7)

Values are mean \pm S.E. Number of observations are shown in parentheses. Glucagon depleted rats had been treated with antidiabetic serum and controls with normal rabbit serum. ^a denotes that the sympathectomized group of rats differed significantly ($p < 0.01$) from the other groups.

¹The lack of decrease of hepatic glycogen concentrations was verified by reanalysis of all liver samples.

The concentrations of glucose (mmol l⁻¹) in blood and of insulin (pmol l⁻¹) and glucagon (pmol l⁻¹) serum: 1 rest (□) and 150 ex cis (▨)

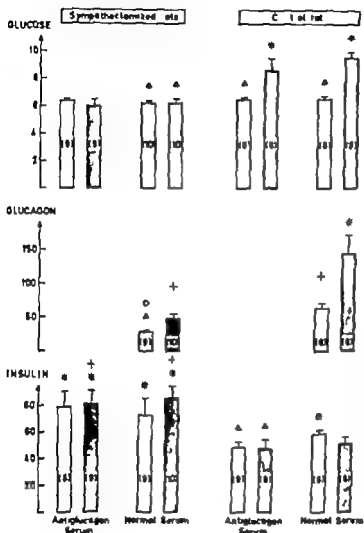


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had increased (Fig. 2 and Table III), whereas the plasma concentrations of FFA, glyceralanine and insulin did not change significantly. During exercise in control rats glucose concentrations correlated significantly with norepinephrine ($r = 0.91$, $p < 0.01$, $n = 7$) as well as with epinephrine ($r = 0.77$, $p < 0.05$, $n = 7$) concentrations in plasma. In none of the rest groups of rats did the blood glucose concentration change significantly during the 90 min rest period (not shown).

Effects of sympathectomy

The concentrations of catecholamines in pancreas, liver and skeletal muscle (Table I) as well as in plasma (Table II) were significantly diminished by the applied combination of chemical sympathectomy and adrenalectomy. The concentrations of epinephrine and norepinephrine in plasma correlated significantly during exercise in control rats ($r = 0.85$, $p < 0.01$, $n = 7$).

of less efficient sympathetomy procedures (Gollnick *et al.* 1970, Maling *et al.* 1966, Sembrowich *et al.* 1974) or of less precise techniques for the determination of glycogen (Maling *et al.* 1966), is the lack of appropriate controls of experiments (Gollnick *et al.* 1970, Sembrowich *et al.* 1974), is the use of trained animals (Gollnick *et al.* 1970, Sembrowich *et al.* 1974) or the employed exercise procedures.

It has been repeatedly observed that glycogen depletion can be elicited in isolated, perfused skeletal muscle by electrical stimulation (Berger *et al.* 1973, Chapter and Sainsby 1968, Ooms *et al.* 1970, Heinrich and Cori 1966, Karlsson, Rosell and Saltin 1972). Furthermore, the capacity for work performance is reduced in patients with McArdle's disease, in whom muscular glycogenolysis is inhibited by a deficiency of glycogen phosphorylase (Pearson, Riser and Mommersaerts 1961). At first thought our present finding that sympathetomized animals were able to exercise for a prolonged period without a fall in muscle glycogen may seem incompatible with these facts. But catecholamines may be present during periods of skeletal muscle preparations either as a consequence of excitation of sympathetic nerve fibers within the electrically stimulated nerve or due to the use of blood as the perfusion medium. Furthermore, insufficient supply of oxygen or free fatty acids to the isolated contracting muscle due to insufficient perfusion flow or to the use of artificial perfusates may enhance glycogenolysis and thus reduce the applicability of findings in isolated preparations to the interpretation of findings in intact exercising animals. As to the limited work tolerance in patients suffering from McArdle's disease it should be re-emphasized that these patients tolerate low work intensities indefinitely (Pearson *et al.* 1961).

It is probably so that very low work loads normally can not elicit muscular glycogenolysis (Chapter and Sainsby 1968). A range of higher work loads including the one used in the present study may be characterized by the existence of a catecholamine facilitated muscular glycogenolysis during physiological conditions and by the ability of the working muscles to derive the necessary energy from alternative sources thus performing equally well when glycogenolysis is impeded. In accordance with these considerations pretreatment of an isolated muscle with epinephrine in concentrations, which per se does not produce a rise in the concentration of active phosphorylase, causes an augmented increase in the concentration of this enzyme during subsequent electrical stimulation (Heinrich and Cori 1966). Furthermore, beta adrenergic blockade has been shown to diminish the reduction of glycogen content in an *in situ* isolated, stimulated muscle preparation (Costin *et al.* 1971). The possibility that breakdown of other substrates may substitute for glycogen breakdown during prolonged exercise is supported by the findings of the present study as well as of the ability of a fat emulsion to improve exercise tolerance in patients suffering from McArdle's disease (Pearson *et al.* 1961) and of the ability of working muscles to utilize endogenous triglycerides during prolonged exercise (Reitman, Baldwin and Holloszy 1973).

As to the pancreatic hormonal response to exercise we found that in control rats, in spite of an increase in blood glucose concentrations, plasma insulin concentrations were unchanged. While glucagon concentrations increased during exercise. In sympathetomized rats no such exercise-induced inhibition of insulin secretion was found, and the exercise-induced increase of glucagon secretion was much less prominent (Fig. 2). In these rats compared to control rats glucagon concentrations increased less and insulin concentrations were

TABLE III The concentrations in serum (FFA and glycerol), blood (lactate and pyruvate), plasma (alanine) and in the deep part of the vastus lateralis muscle (glycogen) of substrates in rats at rest after 75 min of swimming. The rats were either sympathectomized (SX) or controls (C) either treated with antiglucagon serum or normal rabbit serum.

	FFA mEq l ⁻¹	Glycerol mmol l ⁻¹	Lactate mmol l ⁻¹	Pyruvate μmol l ⁻¹	Alanine μmol l ⁻¹	Glycogen mmol l ⁻¹
C-rats, normal serum	1.325 ± 0.186	0.473 ± 0.079	2.66 ± 0.67	68 ± 12	392 ± 15	33 ± 2
Rest	(8)	(8)	(8)	(8)	(7)	(8)
C-rats, normal serum	1.151 ± 0.226	0.427 ± 0.040	5.69 ± 0.63	136 ± 24	353 ± 21	19 ± 2
After exercise	(8)	(8)	(8)	(8)	(8)	(8)
C-rats, antiglucagon serum	1.278 ± 0.284	0.406 ± 0.105	1.71 ± 0.09	74 ± 10	471 ± 36	32 ± 2
Rest	(9)	(9)	(9)	(9)	(8)	(9)
C-rats, antiglucagon serum	1.397 ± 0.306	0.568 ± 0.104	5.32 ± 1.20	163 ± 23	366 ± 21	19 ± 2
After exercise	(6)	(6)	(8)	(8)	(8)	(8)
SX-rats, normal serum	0.644 ± 0.080	0.297 ± 0.034	1.69 ± 0.17	56 ± 14	420 ± 18	40 ± 3
Rest	(9)	(9)	(9)	(8)	(8)	(9)
SX-rats, normal serum	0.799 ± 0.063	0.373 ± 0.036	6.99 ± 1.97	126 ± 18	352 ± 18	40 ± 2
After exercise	(9)	(9)	(8)	(8)	(9)	(10)
SX-rats, antiglucagon serum	1.366 ± 0.190	0.534 ± 0.109	1.54 ± 0.08	53 ± 12	480 ± 32	40 ± 2
Rest	(8)	(8)	(8)	(8)	(8)	(8)
SX-rats, antiglucagon serum	1.042 ± 0.123	0.562 ± 0.063	4.62 ± 1.13	103 ± 15	430 ± 22	38 ± 4
After exercise	(8)	(7)	(8)	(8)	(8)	(8)
Least significant difference	0.455	0.184	2.26	43	57	6

Values are mean ± S.E. Number of observations are shown in parentheses. The least significant difference ($p < 0.05$) between two mean values of the same variable was calculated by analysis of variance and Tukey test (Snedecor and Cochran 1965).

Discussion

The present study has demonstrated a remarkable influence of sympathectomy on metabolic and hormonal responses to exercise. In contrast to findings in control rats neither hepatic nor muscular glycogen concentrations decreased in sympathectomized rats during exercise. Previous studies on the contribution of the sympatho-adrenal system in the glycogen depletion in liver and skeletal muscle during exercise have given inconsistent results. Certainly although less pronounced compared to the one in the present study an inhibition of exercise induced glycogen depletion has been found in some studies of adrenalectomized rats (Sembrowich *et al.* 1973; Struck and Tipton 1974) and of rats adrenalectomized as well as chemically sympathectomized (Maling *et al.* 1966). But other studies of adrenalectomized rats (Gollnick *et al.* 1970; Maling *et al.* 1966) and of rats adrenalectomized as well as chemically sympathectomized (Gollnick *et al.* 1970; Sembrowich *et al.* 1973) failed to demonstrate any inhibition of glycogenolysis. The reasons why these studies in contrast to the present study did not convincingly reveal the significance of an intact sympatho-adrenal system for the glycogen depletion during exercise are likely to be found in the use

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higher in the face of lower blood glucose concentrations during exercise. These findings which could not be ascribed to differences in plasma levels of FFA and alanine (Table III) indicate that in the rat sympathetic discharge significantly promotes glucagon secretion and inhibits insulin secretion during prolonged exercise. Similar conclusions have previously been drawn from studies of rats which before exercise had been either immunosympathectomized, adrenalectomized or treated with adrenergic blocking agents (Brisson, Malaisse-Lagae and Malaisse 1971, Luyckx and Lefebvre 1974, Luyckx *et al.* 1975). Since in man catecholamines probably account to only a minor extent for the glucagon response to prolonged exercise (Galbo *et al.* 1977a, 1977c), a species difference as regards the regulation of glucagon secretion during exercise seems to exist between man and rat.

The lower concentration of glucagon in plasma during exercise after sympathectomy hardly explained the lack of hepatic glycogen depletion in sympathectomized rats. This is so since in glucagon depleted control rats the rate of hepatic glycogen depletion in the absence of a compensating catecholamine stimulus (Table II) were the same as in control rats treated with normal rabbit serum. This apparent lack of influence of glucagon on hepatic glycogen mobilization in exercising rats is inconsistent with the conclusion in a previous study in which hepatic glycogen depletion in control rats was larger than in the present study (Galbo and Holst 1976). However during exercise increased glucagon secretion possibly only enhances hepatic glycogen mobilization at high rates of glycogen breakdown or at reduced glycogen levels. Alternatively the lack of decrease of plasma insulin concentrations during exercise found only in the present study accounts for the discrepancy since insulin deficiency makes the liver more sensitive to the glycogenolytic effect of glucagon (Mackrell and Sokal 1969, Wagle 1975).

The present study has shown that the pancreatic hormonal response as well as muscular and hepatic glycogen depletion during prolonged exercise are markedly reduced in sympathectomized rats compared to control rats. These findings indicate that the sympathetic-adrenal system enhances glucagon secretion and glycogen depletion but inhibits insulin secretion in normal exercising rats. The contributions of sympathetic nervous activity and of adrenomedullary secretion to these effects still have to be settled. Neither has the present study established whether catecholamines directly enhance glycogen breakdown and glucagon secretion during exercise or whether these effects are due to a primary inhibition of insulin secretion (Brisson *et al.* 1971, Galbo *et al.* 1977c, Luyckx and Lefebvre 1974, Porte 1967). Insulin being able to inhibit glucagon secretion (Charles *et al.* 1976) and to promote glycogen storage (Berger *et al.* 1975, Craig, Rall and Lerner 1969, Mackrell and Sokal 1969, Miller and Lerner 1973).

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fluid mobilization from the extra- to the intravascular space (Lundvall and Hillman, 1966b). The described findings suggest that neurogenic and humoral microvascular constrictor effects, if evoked during integrated reflex activation of the sympatho-adrenal system, might be of considerable importance for transcapillary hydrodynamic eq.

A hypothesis was tested in the present study in which the sympatho-adrenal system was activated by hemorrhage. During bleeding, transcapillary absorption of fluid into the blood constitutes an important mechanism for refill of the circulatory system and thereby for maintenance of cardiovascular homeostasis. Such fluid transfer from the extravascular to the intravascular space mainly occurs in skeletal muscle, which, due to its large tissue mass, contains considerable volumes of fluid to be mobilized into the circulation. The fluid absorption from skeletal muscle during bleeding seems mediated mainly by two mechanisms, *i.e.* by a decrease of the pre- to postcapillary resistance ratio, leading to fluid absorption via a decrease of capillary hydrostatic pressure (*e.g.* Öberg 1964), and by osmotic absorption caused by arterial hyperosmolality due to glucose release from the liver (Lundvall *et al.* 1972, Järnåhr 1973). The relative importance of these two mechanisms has not been more precisely defined. There is experimental evidence to indicate, however, that the direct adrenergic mechanism is the more important one in early stages of bleeding, only at small and moderate blood loss (Öberg 1964), whereas during more prolonged severe hemorrhage the glucose-osmotic fluid absorption seems to be at least equally important (Järnåhr 1973, Järnåhr *et al.* 1976). In the present investigation, in which emphasis was placed on the direct adrenergically evoked fluid mobilization from skeletal muscle during bleeding, the animals were exposed to a relatively small hemorrhage (withdrawal of 15% of blood volume) and the period of hemorrhagic hypotension was limited (10 min) to avoid activation of the glucose-osmotic fluid absorption mechanism.

Methods

Animals of both sexes, weighing 2.4–3.9 kg were used in the study. The animals were anesthetized with sodium pentobarbital (50 mg/kg b.wt.) and urethane (100 mg/kg b.wt.) after induction with ether. Body temperature was maintained at $38 \pm 0.5^\circ\text{C}$ throughout the experiment. An incision was performed on the innermost vascular bed of the lower leg muscles, isolated so that the popliteal artery and vein formed the sole vascular connections with the main part of the animal. In most experiments the muscle preparation was made on the right lower leg only. In some of the animals, also the left lower leg muscles were isolated so that simultaneous observations could be made on two separate leg preparations. The muscle preparation was placed in a water-filled plethysmograph (38°C) to permit continuous recording of tissue volume (for surgical and technical details, see Lundvall 1972). After heparinization (0.5 mg/kg b.wt.), the popliteal vein was cannulated and the venous outflow from the region shunted into a femoral cannula connected to the right jugular vein. An optical drop recorder port was inserted in the venous cannula for continuous recording of blood flow. The arterial inflow pressure was measured from the brachial artery. The right brachial artery was cannulated to permit bleeding and withdrawal of arterial blood for determination of plasma osmolality and plasma glucose concentration. Sampling of venous blood for osmolality and glucose determination was made via a T-tube in the venous cannula close to the cannulated femoral vein. Plasma osmolality was measured by thermistor cryoscopy (Osmometer 31 LAS, Advanced Instruments, Inc.) and plasma glucose concentration with the glucose-oxidase method. Blood flow and arterial blood pressure, the latter measured with a Statham transducer were recorded on Grass Polygraph. The sections of the resistance vessels in the muscle caused by bleeding were obtained from the regional intra-vascular pressure difference and blood flow. Net transcapillary fluid movements were determined

Fluid transfer from skeletal muscle to blood during hemorrhage

Importance of beta adrenergic vascular mechanisms

By

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Abstract

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Vascular reactions in the cat lower leg muscles in response to short term (10 min) hemorrhagic hypotension (~ 80 mmHg) were studied before and after regional blockade of the β -adrenoceptors. In the muscle region with intact β -adrenoceptors, hemorrhage raised vascular resistance by about 80% and caused a dilatation of the precapillary sphincters, the latter effect evidenced in terms of a 35% increase of the capillary filtration coefficient. Concomitantly an absorption of extravascular fluid to the blood stream occurred, a process tending to compensate for the reduction of intravascular fluid volume. After regional β -blockade there was quite a marked augmentation of the hemorrhage induced increase of vascular resistance whereas the inhibition of precapillary sphincter tone and the transcapillary fluid absorption were almost abolished. These observations indicate that bleeding is associated with a significant β -adrenergic dilator influence in both the resistance vessels and precapillary sphincters of skeletal muscle and that the β -dilator mechanism may be essential for the important, compensatory fluid gain from the extravascular to the intravascular space during hemorrhage. The observed β -adrenergic mediation of the net transcapillary fluid absorption could be ascribed to resetting of the pre/postcapillary resistance ratio, leading to decreased capillary hydrostatic pressure, and to the dilator influence in the precapillary sphincters, leading to an increased number of the patent capillaries available for the transcapillary fluid exchange.

Recent studies from this laboratory have demonstrated that electrical activation of the sympathetic vasomotor fibres to skeletal muscle is associated with a β -adrenergic dilator response in resistance vessels of small diameter and in precapillary sphincters (Lundvall and Järhult 1974, 1976). The β -adrenergic dilator component of the sympathetic vascular response was found to markedly counteract the α -adrenergic constriction in the microcirculation and to significantly augment the net transcapillary absorption of extravascular fluid induced by sympathetic stimulation. The facilitation of the fluid transfer could be ascribed partly to the inhibition of precapillary sphincter tone, leading to an increased number of patent capillaries available for transcapillary exchange, and partly to a reinforcement of the nerve induced fall of capillary hydrostatic pressure. Intra-arterial infusion of adrenaline and noradrenaline was found to cause similar preferential microvascular β -adrenergic dilator effects contrib-

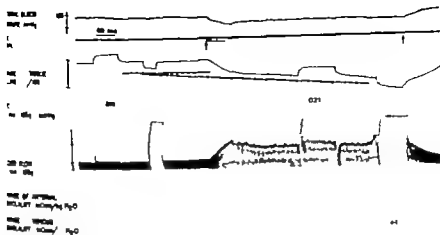


Fig. 1. Original record illustrating the pattern of vascular response evoked by bleeding. Onset of hemorrhage and increase of blood indicated by arrows. Changes of plasma osmolality given at bottom of the record. A detailed description see text.

gradually steady state level of $3.7 \text{ ml/min } 100 \text{ g}$. The flow reduction was partly due to a pressure fall and partly to an increase of regional vascular resistance, which was raised from the control value of 11.6 to $21.4 \text{ mmHg/(ml/min } 100 \text{ g)}$ in the steady state period of bleeding. Tissue volume, being approximately constant in the control period (isovolumic bar), showed quite a pronounced decrease initially during hemorrhage reflecting mobilization of regional blood (capacitance response). The subsequent slow and continuous decline of tissue volume, more clearly visualized by the broken lines drawn beneath the volume recording, represents net transcapillary fluid absorption, in this experiment occurring at a rate of $0.055 \text{ ml/min } 100 \text{ g tissue}$. The CFC values obtained in the record indicate that bleeding was associated with a relaxation of the precapillary sphincters, which increased the luminal capillary surface area for fluid absorption by some 50% (cf. Methods). The figures for plasma osmolality at bottom of the record, finally show that bleeding caused some increase ($1 \text{ mOsm/kg H}_2\text{O}$) of arterial osmolality and that venous osmolality also tended to increase.

Regional blockade of the β -adrenoceptor was found to cause quite marked quantitative deviations of the described vascular responses evoked by hemorrhage. The resistance increase evoked during bleeding was thus always greater after β -blockade, whereas the inhibition of precapillary sphincter tone and the net transcapillary fluid absorption instead was much reduced. These effects are summarized in Fig. 2 (mean values \pm S.E.). Panel A shows that vascular resistance in the intact region was raised during hemorrhage by 77% above the resting control level whereas in the β -blocked region the corresponding resistance increase averaged 112% ($p < 0.05$). This augmentation after β -blockade indicates the presence during hemorrhage of a β -adrenergic dilator influence, strong enough to significantly counteract the simultaneous constrictor response in the resistance vessels. The changes of arterial blood pressure during bleeding were quite similar before and after β -blockade;

from observed alterations of tissue volume and could be separated from capacitance effects in the vascular bed, since the latter develop rapidly and in coordination with the effects in the resistance vessels (see Linder 1960, Kjellmer 1965). Precapillary sphincter activity determining the number of patent capillaries and, hence, the functional capillary surface area available for transcapillary exchange, was followed by measurement of the capillary filtration coefficient (CFC). CFC was determined from the tissue volume coding by calculating the rate of net transcapillary fluid filtration caused by raising venous outflow pressure (VP) 5 mmHg, assuming that 80% of the venous pressure rise was transmitted to the capillary level (Cobbold *et al.* 1963). Venous pressure was changed by adjustment of the height of the orifice of the outflow tubing from the muscle region. Except during the CFC determination, venous pressure was maintained at a normal level (4–6 mmHg), which gave a constant tissue volume in the control period (isovolumic state).

When the surgical procedures were finished the animals rested for 30 min before experimental intervention. Hemorrhage was performed by bleeding the animal into a graded syringe so that 15% of the calculated blood volume was withdrawn within 1 min (blood volume considered to be 7% of the body weight, Alon and Dittmar 1971). After 10 min of hemorrhage the shed blood was reinfused. Regional β -adrenoceptor blockade was produced by administration of propranolol (600 μ g/kg muscle) close arterially via a cannula inserted into a side branch of the femoral artery. The venous outflow was discarded during the administration of propranolol to avoid systemic effects. Test experiments in which the dextro-isomer of propranolol was used showed that the reported effects of the blocking agent were not related to a nonspecific inhibition, such as an anesthetic one.

In Results section, spread of data is expressed as S.E. Significance tests were performed according to Student's *t* test.

Results

The effect of regional blockade of the β -adrenoceptors on the vascular responses evoked by hemorrhage in the innervated lower leg muscle preparation was studied in 9 animals. In 6 of the animals observations were made on the vascular bed of the right lower leg muscles only. The vascular changes induced by standardized bleeding (withdrawal of 15% of the blood volume for a period of 10 min) were here first studied when the β -adrenoceptors were intact (below denoted "intact" region). After reinfusion of the shed blood, regional blockade of the β -adrenoceptors was performed and the hemorrhage experiment repeated after a 2 min period of rest. Separate control experiments on 4 animals in which no β -blockade was performed showed that repetitive bleeding as such evoked reproducible changes. In the other 3 animals in which the effect of β -blockade was studied, the lower leg muscles were isolated on both sides and after the 30 min period of rest after completion of surgery propranolol was given close arterially to the right leg muscles whereas the left leg muscle preparation was left intact. This experimental approach permitted simultaneous observations of the vascular responses in the intact and in the β -blocked muscle region during hemorrhage. Two periods (10 min) of bleeding with an interval of 20–30 min were performed on each of these 3 animals. The results from the described two groups of animals in which β -blockade was performed were quite similar and will be presented together.

Fig. 1 showing tracings of mean arterial blood pressure, muscle blood flow and tissue volume, illustrates the typical pattern of vascular response evoked by hemorrhage in the intact muscle region. The capillary filtration coefficient (CFC) and arterial and venous osmolality were determined before and during bleeding. It can be seen that hemorrhage caused a rapid decrease of arterial blood pressure down to an initial, transient low level (65 mmHg), after which pressure increased somewhat and stabilized on a hypotension level (~80 mmHg) maintained throughout the period of bleeding. Concomitant to the pressure decline, blood flow decreased rapidly from the control value of 8.8 ml/min/100 g to an

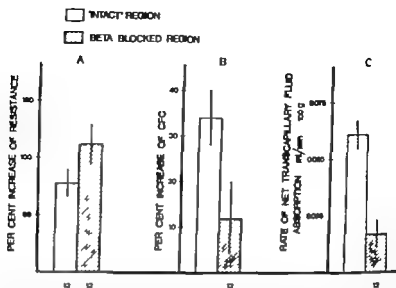


Fig. 2. Diagrams showing the effects of bleeding on vascular resistance (Panel A), on CFC, reflecting precapillary sphincter tone (Panel B), and on net transcapillary fluid absorption (Panel C) before and after β -blockade. The data shows that β -blockade augmented the resistance increase during hemorrhage where the precapillary sphincter dilatation and transcapillary fluid transfer were markedly reduced. The conclusion was reached that hemorrhage is associated with a β -adrenergic dilator influence in the resistance vessels or precapillary sphincters and that the β -dilator effects are essential for the gain of extravascular fluid to the blood stream.

control blood pressure averaged 117 mmHg in the intact region and 118 mmHg in the β -blocked region and the steady state hemorrhage hypotension averaged 77 and 75 mmHg respectively. Resistance changes due to passive elastic recoil of the vascular wall as transmural pressure decreased must therefore have been in the same order of magnitude in the two situations.

CFC in the resting control period averaged 0.013 ± 0.001 ($n=12$) ml/min 100 g mmHg in the intact region and 0.015 ± 0.001 ($n=12$) ml/min 100 g mmHg in the β -blocked region. Panel B in Fig. 2 shows the mean per cent increase of CFC above control during bleeding. It can be seen that in the intact region, CFC was raised by 34%. This effect, reflecting a relaxation of the precapillary sphincters, seems to a great extent caused by β -adrenoceptor activation since the increment of CFC in response to hypovolemia in the propranolol treated muscle was much smaller (12% $p < 0.01$).

Diagram C in Fig. 2 depicts the rate of net transcapillary fluid absorption during hypovolemia. The data refer to observations 8–9 min after commencement of bleeding but are representative for the whole period of bleeding since the absorption rate was roughly constant in the intact (cf Fig. 1) as well as in the β -blocked region. In the intact muscle region, the rate of fluid transfer averaged 0.962 ± 0.006 ($n=12$) ml/min 100 g tissue implying that a total of some 0.60 ml/100 g tissue of extravascular fluid was mobilized into the blood stream during the 10 min period of hypovolemia. The observed fluid transfer can mainly be ascribed to a reflex decrease of capillary hydrostatic pressure since the increment of arterial blood osmolality during bleeding, leading to osmotic fluid gain into the circulatory system, was quite small (see Discussion). Arterial plasma osmolality was raised by 4 mOsm/kg H_2O on an

slope towards the end of the period of hemorrhage. Concomitantly venous osmolality increased by a mean value of 2 mOsm/kg H_2O . The determinations of plasma glucose concentration indicated that both the arterial and venous osmolar increase almost entirely was due to hyperglycemia (1 mM/L of glucose considered to correspond to 1 mOsm/kg H_2O), a finding in agreement with previous reports (Järhult 1973, Järhult *et al* 1976).

In the β -blocked muscle region, the rate of fluid absorption was much reduced compared to that in the intact region ($p < 0.001$) and it here averaged only 0.017 ± 0.005 (n = 12) ml/100 g tissue (corresponding to a total amount of mobilized fluid during the 10 min of bleeding of about 0.15–0.20 ml/100 g tissue). This reduction of the fluid transfer after β -blockade can hardly be related to the glucose-osmotic mechanism since the observed alterations of blood osmolality during bleeding was similar to that in the experiments in which the β -receptors in the muscle region were left intact. Arterial osmolality thus showed an average increase of 3 mOsm/kg H_2O during bleeding and venous osmolality concomitantly rose by 1 mOsm/kg H_2O as in the intact region, the osmolar change could be ascribed to an increase of the plasma glucose concentration. The effects of propranolol on the fluid transfer during hemorrhage instead seems related to blockade of regional, vascular β -adrenergic mechanisms in the muscle which, in the intact region, facilitate transcapillary fluid mobilization. β -adrenergic relaxation of precapillary sphincter tone, increasing the number of patent capillaries and thereby the capillary surface area available for transcapillary fluid exchange, seems to be one such effect as indicated by the above described CFC data.

Discussion

The present study strongly suggests that bleeding is associated with a β -adrenergic dilator influence in the skeletal muscle vascular bed. This was revealed in terms of a significantly higher vascular tone in both the resistance vessels and in the precapillary sphincters in the β -blocked muscle region than in the muscle region with intact β -adrenoceptors during standard hemorrhagic hypotension. It should be stressed that the alterations of the vascular responses to bleeding evoked by β -blockade entirely must reflect a regional effect in the skeletal muscle of the β -blocking agent. The β -blocking agent was thus administered locally by close arterial infusion and concomitantly the venous effluent was sampled and discarded. Interference of the remote nervous and hormonal control of the muscle vascular bed due to, for example, blockade of β -adrenoceptors in the central circulation or possible interference with autonomic circulatory control centres was thereby avoided.

As a result of the β -adrenergic dilator influence, the net resistance increase in the muscle during bleeding was reduced by about 30%. In absolute resistance units, the β -adrenergic effect amounted to some 5 mmHg/(ml/min 100 g). This quite pronounced effect in skeletal muscle, one of the main targets for vasomotor control concerned with the maintenance of general cardiovascular homeostasis, suggest that β -adrenergic inhibition of vascular tone must be taken into account in studies aimed to investigate the control of the resistance function in bleeding, a research field which currently receives great interest. The inhibition of precapillary sphincter tone during hypovolemia has been attributed to the action of "vasoconstrictor metabolites" accumulated as a result of the reduced blood flow (e.g. Lewis and McIsaac 1962, Lundgren, Lundvall and Mellander 1964, Öberg 1964). The present findings

indicate that this response, which might be considered an important compensatory mechanism facilitating capillary exchange, instead might be mainly mediated by β -adrenoceptor activation at least during bleeding of short duration. It is possible, however, that the relative importance of "vasodilator metabolites" for the precapillary sphincter dilatation will be greater at more severe hemorrhage due to the attendant, marked decrease of regional blood supply. Furthermore, vascular transmural pressure in the sphincter section may decrease significantly during pronounced hemorrhagic hypotension and cause a myogenic inhibition of precapillary sphincter tone (cf. Cobbold *et al.* 1963). Yet, preliminary data from an extended series of experiments in which graded bleeding was performed indicate that the adrenergic mechanism is mainly responsible for the inhibition of precapillary sphincter tone also during severe hemorrhage, and, further, that the β -adrenergic dilator influence in resistance vessels during bleeding can be even more pronounced than in the present study.

The β -adrenergic dilator influence in the muscle vascular bed during hemorrhage may be both neurogenic and humoral in origin. Sympathetic activation of the skeletal muscle vascular bed has been shown to produce a neurogenic β -adrenergic dilator response markedly affecting the precapillary sphincters and the blood flow distribution in the muscle (Vibero, Click and Renkin 1968; Lundvall and Järhult 1974, 1976). This neurogenic β -dilator mechanism seems, however, of little importance for the volume flow of blood as regulated by the muscle vascular resistance (Lundvall and Järhult 1976). The observed β -adrenergic inhibition of vascular tone in the resistance vessels may instead be mainly related to the greatly increased release of adrenaline from the suprarenal glands during bleeding (e.g. Watts 1965; Hall and Hodge 1971; Jakachik *et al.* 1974), an assumption supported by some preliminary experiments in which hemorrhage was performed before and after removal of the adrenals. The blood-borne catecholamines may also contribute to the dilation of the precapillary sphincters during bleeding as suggested by infusion experiments (Lundvall and Hillman, to be published). — The question may be raised in this context whether the observed β -adrenergic dilator effects in the resistance vessels and precapillary sphincters during hemorrhage reflect direct interaction with the specific receptors of the vascular smooth muscle or if they are indirect and secondary to alterations of the skeletal muscle metabolism produced by adrenergic substances. This problem, the site of action of catecholamines in vasodilatation has since long been a matter of debate and the present results do not permit any conclusion to be drawn. With regard to skeletal muscle, however, current views seem to strongly favor the mechanism of direct stimulation of β -receptors of the vascular effector cells. The recently described β -adrenergic facilitation of transmitter release from the adrenergic nerve endings (cf. Langer 1977) also deserves consideration when discussing the site of action of the adrenoceptor mediated inhibition of vascular tone during hemorrhage. Blockade of any α -presynaptic β -effect would, in fact, tend to diminish the recorded increase of vascular tone in the resistance vessels and precapillary sphincters after administration of propranolol, thereby leading to an underestimation of the postsynaptic β -adrenergic dilator influence.

The most prominent alteration after β -blockade of the vascular response to bleeding was the pronounced reduction of the net transcapillary fluid absorption. Two main mechanisms have been proposed to be responsible for the important, compensatory fluid transfer from the interstitial to the intravascular compartment during hypovolemia. The hemorrhage induced

loss of the sympatho-adrenal activity elicits a resetting of the pre to postcapillary resistance leading to decreased capillary hydrostatic pressure and thereby to fluid absorption (Öberg 1964) and arterial hyperosmolality due to glucose release from the liver and osmotic fluid absorption (Järhult *et al.* 1972, Järhult 1973). The observed reduction of the fluid transfer in response to β -blockade would *a priori* seem related to an interference of the blocking agent with the adrenergically evoked fluid transfer—an assumption supported by the fact that the osmolar changes in the blood during the present relatively mild and slow-rate bleeding was small and quite similar before and after β -blockade. The contribution of osmotic fluid transfer to the observed total fluid absorption observed may in fact be assessed from the measured osmolar changes and blood flow. Thus, a previous experimental study (Lundvall 1972) showed that non-protein osmotic fluid transfer in the cat lower leg muscle, being flow dependent, is directly proportional to the osmolar uptake in the muscle, an experimental finding supported by theoretical analyses (Lundvall 1972, Salathe and An 1976). Experiments in which hypertonic glucose was administered i.v. so as to produce an arterial hyperglycemia and hyperosmolality in the range encountered in bleeding, i.e. 0–30 mOsm/kg H₂O (*cf.* Järhult *et al.* 1976), confirm the mentioned previous observations and show that the glucose osmotic fluid flux (ml/min \cdot 100 g) in cat muscle can be calculated from the following numerical product volume flow of blood (in ml/min \cdot 100 g) the arterio-venous osmolar difference (in mOsm/kg H₂O) $1.75 \cdot 10^{-7}$ (Lundvall, unpublished *cf.* also Lundvall 1972). Using this formula, the average rate of osmotic fluid transfer at the time of fluid sampling for osmolality determination in the present hemorrhage expts. would amount to 0.003 ml/min \cdot 100 g in the intact muscle region and to 0.012 ml/min \cdot 100 g in the β -blocked region. Subtraction of these values from the observed rates of fluid transfer during bleeding indicates that, in the intact region, net transcapillary fluid absorption due to decreased capillary hydrostatic pressure amounted to 0.049 ml/min \cdot 100 g, corresponding to 8% of the total fluid gain, and that it was negligible in the β -blocked region. These deductions indicate that the fluid transfer from skeletal muscle to the blood stream in the present hemorrhage expts. mainly was due to reflex adrenergic mechanisms and that β -adrenoceptor stimulation was essential for the adrenergically evoked fluid gain.

It seems likely that the β -adrenergic mediation of fluid transfer from muscle to blood during hemorrhage is causally linked to the observed β -dilator vascular effects and that it reflects the neurogenic and humoral β -adrenergic facilitation of hydrodynamic transcapillary exchange in skeletal muscle that can be observed during sympathetic stimulation and during infusion of adrenaline and noradrenaline (Lundvall and Järhult 1976, Lundvall and Hillman, *in press*). The β -adrenergic inhibition of precapillary sphincter tone, leading to an increased number of patent capillaries and, hence, to an increased capillary surface area available for transcapillary fluid exchange, must have contributed significantly to the observed, pronounced β -adrenergic facilitation of the fluid transfer in bleeding. It can be deduced, however, that a β -adrenergic adjustment of the pre to postcapillary resistance also leading to a fall of capillary hydrostatic pressure, must have been at least equally important as the precapillary sphincter dilatation. Such a resetting of the pre to postcapillary resistance ratio indicates that the β -adrenergic dilator influence, in relative terms, was more pronounced in the postcapillary than in the precapillary resistance vessels.

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Vasodilatation and modulation of vasoconstriction in canine subcutaneous adipose tissue caused by activation of β -adrenoceptors

By

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Abstract

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The present experiments were undertaken to study the balance between vascular α - and β -adrenoceptors in canine subcutaneous adipose tissue during sympathetic nerve stimulation and noradrenaline injections. Propranolol potentiated and prolonged the vasoconstrictor response to close i.a. injections of noradrenaline. The vasoconstriction induced by brief nerve stimulation (0.5 to 8 Hz) was, however, unaltered by the β -adrenoceptor blockade. During prolonged nerve stimulation the vasoconstrictor response was well sustained at 1-3 Hz but at 4 Hz there was gradual escape. The escape phenomenon at 4 Hz was abolished by propranolol. The β_1 -selective antagonist practolol, like propranolol, potentiated and prolonged the vasoconstriction induced by noradrenaline injections and reduced the vasoconstrictor escape during prolonged nerve stimulation at 4 Hz. Furthermore, the vasodilatation induced by noradrenaline during prolonged nerve stimulation during α -adrenoceptor blockade was abolished by practolol. Practolol also blocked the lipolytic response to noradrenaline and nerve stimulation. The β_2 -selective antagonist H35/25 blocked the effects of the β_1 -selective partial antagonist but failed to alter noradrenaline as well as nerve stimulation induced vascular and lipolytic β -adrenoceptor responses. The present results provide further support for the hypothesis that vascular β -adrenoceptors in adipose tissue are innervated (noninnervated), predominantly activated by circulating noradrenaline. Moreover, both vascular and lipolytic β -adrenoceptors activated by noradrenaline in adipose tissue are best classified as β_2 -adrenoceptors.

The lipolytic response to intravascularly administered noradrenaline as well as that of noradrenaline released from the adrenergic nerve terminals in canine subcutaneous adipose tissue is mediated via activation of β -adrenoceptors (Fredholm and Rosell 1968, Ballard *et al.* 1971). The vascular responses to these stimuli are however qualitatively different. Thus, noradrenaline released from nerve terminals invariably causes α -adrenoceptor mediated vasoconstriction (Ngai *et al.* 1966, Hoffbrand and Forsyth 1973) whereas intravascular noradrenaline may induce vasodilatation (Nilsen *et al.* 1968, Ballard *et al.* 1971, Hoffbrand and Forsyth 1973). The explanation for these differences in vascular responses may be that the vascular α -adrenoceptors are innervated receptors in close contact with the sympathetic nerve endings whereas the vascular β -adrenoceptors are humoral (noninnervated).

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usually is well known to be highly selective for β_1 - and β_2 -adrenoceptors respectively (Levy and Walzshaid 1967). In each experiment a control treatment with the chosen stimulus—i.e. sympathetic nerve stimulation, noradrenaline infusion I.A. or subarterial infusion I.A.—was first applied and blood flow and glycerol release are followed. Either practolol or H13/75 was then administered and the next stimulus was applied after a period of 30–40 min to permit distribution of the antagonist. In some experiments, the procedure was then used to study the other antagonist in the same manner.

Sympathetic nerve stimulation was performed either for 30 min at 4 Hz or after α -adrenoceptor blockade with hydralazine (200 μ g i.v.), for 4 min at 2 Hz. Noradrenaline was injected close I.A. (10^{-6} – 10^{-9} mol). After hydralazine treatment noradrenaline was infused at 2.3×10^{-9} mol per min in 6 rats giving a calculated blood concentration of 4.1×10^{-9} M. Salbutamol was infused at 1.4 to 1.8×10^{-6} mol per min in 8 rats giving a calculated blood concentration of 3.2 to 3.4×10^{-6} M. When these infusions of noradrenaline or salbutamol were performed the amount of agonist per infusion was the same throughout each experiment. Since the blood flow decreased gradually in most experiments the blood concentrations of noradrenaline and salbutamol were usually highest during the latest infusion.

Estimation of data

Flow reduction was calculated from the per cent decrease in resistance ($\text{mmHg } 100 \text{ g min}^{-1} \text{ ml}^{-1}$) and vasoconstriction from the per cent decrease in conductance ($\text{ml min}^{-1} 100 \text{ g}^{-1} \text{ mmHg}^{-1}$). Thus, both vasoconstrictions and vasodilatations are expressed in percentages between 0 and 100. Lipolysis was measured as the net release of glycerol ($\text{nmol } 100 \text{ g}^{-1}$) by subtracting the basal glycerol release from the total release obtained during and after injection of drug or nerve stimulation. Statistical evaluation of the results as performed with Student's *t*-test for paired observations. The following drugs were used: Noradrenaline (1-cresanol HCl, SIGMA), Salbutamol (Salbutamol Sulphate, Allen and Hanbury), Practolol (Glaxo), H13/75 Hünne, Propranolol (Inderal ICI), Hydralazine® (Beckman).

Results

Effect of β -adrenoceptor blockade on vasoconstriction

The vasoconstriction in adipose tissue induced by sympathetic nerve stimulation at 2 Hz was similar on both the control and the propranolol-treated sides, but the vasoconstriction induced by noradrenaline (10^{-6} mol I.A.) was increased and prolonged by propranolol pretreatment (Fig. 1). The reactive hyperaemia after the nerve stimulation period was greater on the control side than on the treated side. Isoprenaline (10^{-10} mol I.A.) caused vasodilatation only on the control side demonstrating a high degree of β -adrenoceptor blockade on the propranolol treated side.

The dose effect relationships from all experiments are shown in Fig. 2. The peak vasoconstriction induced by noradrenaline was increased in the propranolol treated side in comparison to control. No difference in vasoconstriction response between the two sides was however observed following sympathetic nerve stimulation. These results indicate that the vascular response during a 4 min nerve stimulation is due to activation of α -adrenoceptors only whereas intraarterial noradrenaline activates both α - and β -adrenoceptors simultaneously.

Effect of β -adrenoceptor blockade on vasoconstriction produced by prolonged nerve stimulations

Although there was no apparent activation of vascular β -adrenoceptors during nerve stimulation for 4 min the possibility of β -adrenoceptor activation during longer stimulation periods had to be considered. The vasoconstriction induced by sympathetic nerve stimulation at 13 Hz was sustained during the whole 30 min period of stimulation and of similar size both

vated) receptors preferentially accessible to circulating noradrenaline (Rosell and Belfrage 1975 Belfrage and Rosell 1976)

After α -adrenoceptor blockade, sympathetic nerve stimulation causes vasodilatation by stimulation of unmasked β -adrenoceptors (Ngal *et al.* 1966). It is likely that stimulation of these β -adrenoceptors reduces or modifies the vasoconstrictor response to sympathetic nerve activity in the absence of α -adrenoceptor blockade. This has, however not yet been experimentally verified and the first aim of this investigation was to study the balance between vascular α and β -adrenoceptors, both during sympathetic nerve stimulation and during intraarterial administration of noradrenaline.

Most vascular β -adrenoceptors have been suggested to be of the β_2 -type (Lands *et al.* 1967) and noradrenaline, being predominantly a β -agonist, would thus be expected to have weak actions on vascular β -adrenoceptors. However the β -adrenoceptors mediating vasodilatation in adipose tissue seems to be more sensitive to noradrenaline than those of other tissues (Hofbrand and Forsyth 1973 Hjemdahl and Fredholm 1976). Thus, vascular β -adrenoceptors in adipose tissue may be of the β_1 rather than the β_2 -type. The second aim of this investigation was to examine closely the classification of both vascular and lipolytic β -adrenoceptors in adipose tissue, according to the β_1 and β_2 -adrenoceptor concept.

Methods

The experiments were performed on female mongrel dogs weighing between 6–70 kg. The dogs were anesthetized with sodium pentobarbital (25–30 mg/kg with supplements when necessary). All dogs were given heparin (2 500 units v./kg kindly supplied by AB Vitrum) to prevent blood clotting. The dogs were mechanically ventilated through tracheotomy. The inguinal subcutaneous adipose tissue on both the right and the left side were isolated and autoperfused with blood from a femoral artery (Rosell 1966). Blood flow was measured with an arterial drop recorder and registered together with the blood pressure recorded from carotid artery on JB Orm's Polygraph. The vein draining the adipose tissue was also cannulated in some experiments and a three way stop cock permitted sampling of venous blood for glycerol analyses (Laurell and Tibbling 1966). The adipose tissue nerve, containing sympathetic fibers, was sectioned in all experiments. In those cases where the effects of sympathetic nerve stimulations were studied, the nerve was placed on a bipolar electrode and stimulated at supramaximal intensity (15V) and duration (2 ms).

Effect of β -adrenoceptor blockade on vasoconstriction induced by sympathetic nerve stimulation and intra-arterial noradrenaline injections

The adipose tissue on one side was initially treated with 500 μ g propranolol i.a. and 100–200 μ g were repeatedly given to assure a complete β -adrenoceptor blockade. The venous blood was collected for 3–5 min after each injectio and discarded to minimize the systemic dose of propranolol. Both sides were simultaneously stimulated at the same frequency or treated with the same dose of noradrenaline and the untreated adipose tissue preparation served as control. The parameter studied was the peak vasoconstriction induced by sympathetic nerve stimulation (0.5–8 Hz) for 4 min or by close intra arterial injections of noradrenaline (10^{-8} – 10^{-4} mol). Data for frequency and dose response curves were obtained from 5 dogs.

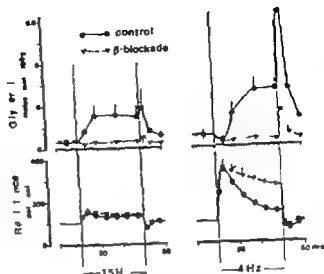
Effect of β -blockade on prolonged (30 minutes) sympathetic nerve stimulations

In 4 dogs one adipose tissue preparation was stimulated at 1.5 Hz for 30 min and the other simultaneously stimulated at 4 Hz for the same period of time. 500 μ g propranolol i.a. was then administered to both sides and the stimulations repeated. Blood flows and glycerol release were followed.

Effect of practolol and H35/25 on the vascular and lipolytic effects of nerve stimulation, subarterial and intra-arterial noradrenaline

In these studies practolol 1 mg/kg i.v. was used as β_1 -adrenoceptor blocking substance and H35/25 2 mg/kg i.v. as a β_2 -adrenoceptor blocking substance. Practolol and H35

Fig. 1. Time course of vasoconstriction and glycerol release during and following sympathetic nerve stimulation for 30 min. The figures are the mean \pm S.E. from 4 dogs. Black dot (•) indicates significant difference between pre-stimulus (β -blockade) and control.



cine antagonist practolol (1 mg/kg i.v.) both the vasodilator and the lipolytic response butanol are unaltered. However practolol inhibited lipolysis as well as the vasoconstriction escape during the nerve stimulation. When the treatments were repeated a time after administration of the β_1 -selective antagonist H15/25 (2 mg/kg i.v.) there is no further alteration of the effects to sympathetic nerve stimulation whereas both vasoconstriction and lipolysis induced by salbutamol were inhibited. This pattern of agonist-antagonist interaction was found in all experiments both for vasodilatation and lipolysis. The vasodilator component in the response to i.a. injection of norepinephrine as well as

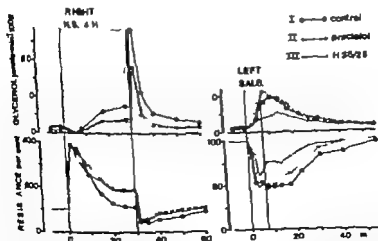


Fig. 2. Time course of the vascular and lipolytic effects of sympathetic nerve stimulation (for 30 min at 15 Hz) in the right side and of i.a. infusion of salbutamol (2.0×10^{-6} mol during 7 min) to the left side subcutaneous adipose tissue in one dog. Treatments were induced three times consecutively: I control II 30 min after administration of practolol (1 mg/kg i.v.) and III 30 min after administration of H15/25 (2 mg/kg i.v.).

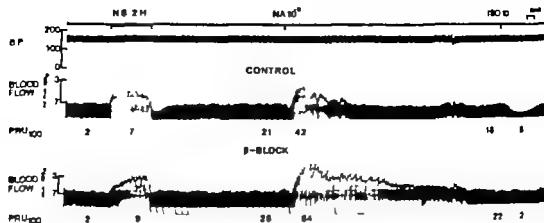


Fig. 1 Effect of propranolol on vascular responses to sympathetic nerve stimulation for 4 min at 2 Hz (N.S.). I.a. injection of noradrenaline 10^{-6} mol (NA) and I.a. injection of isoprenaline (ISO), in two simultaneously perfused adipose tissue preparations in one expt. Recordings are as follows from the top: time scale, systemic blood pressure (BP) and blood flow on the control and the propranolol treated (β -block) side. Values for calculated resistance in PRU₁₀₀ (BP/Blood flow) are given at intervals below each blood flow tracing.

before and after propranolol treatment (Fig. 3). Stimulation at 4 Hz caused the same peak vasoconstriction on both sides but on the control side the response gradually diminished during the 30 min-period of stimulation. This escape was reduced on the propranolol treated side. The lipolysis induced by the 1.5 Hz stimulation was almost the same as that induced by 4 Hz. Propranolol completely inhibited lipolysis induced by both frequencies.

The effect of β_1 and β_2 -selective antagonists

Fig. 4 shows results of an expt. in which salbutamol ($2.0 \cdot 10^{-6}$ moles) was infused to the left adipose tissue preparation for 7 min and where the nerve to the right side tissue was stimulated for 30 min at 4 Hz. When these treatments were repeated after administration of the

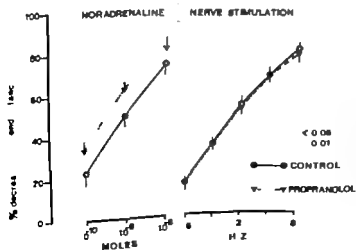


Fig. 2. Effect of β -adrenoceptor blockade with propranolol on peak vasoconstriction to noradrenaline and to sympathetic nerve stimulation for 4 min. Figures are given as mean \pm S.E. from 5 dogs.

TABLE III. Effect of β -adrenoceptor blockade on lipolysis in 9 dogs. The net release of glycerol after surgical treatment are given. When both β -blockers were used H35/25 was administered prior to practolol except in expt. 5 as indicated. SALB = 1 μ l. infusion of salbutamol, N.E. = sympathetic nerve stimulation, NA = i.a. injection or infusion of noradrenaline.

Expt. no.	Treatment	Net glycerol release (μ mol 100 g ⁻¹)		
		Control	H35/25	Practolol
1	SALB	42.8	—	35.9
2	SALB	5.2	0.3	3.8
3	SALB	70.7	15.4	37.4
	N.E.	62.5	30.2	10.8
4	SALB	13.5	2.2	6.2
	N.E.	24.8	17.1	6.8
5	SALB	89.9	33.0*	68.6
	N.E.	116.0	31.5	27.6
6	N.E.	79.6	—	23.7
	N.E.	53.0	—	9.9
	NA	17.4	—	3.3
	NA	2.9	—	0
7	NA	5.5	—	0.5
	NA	40.2	—	14.9
8	NA (α -blockade)	3.8	—	1.2
	N.E. (α -blockade)	2.0	—	0.2
9	NA (α -blockade)	7.5	9.8	0
	N.E. (α -blockade)	9.8	12.2	1.8

*Indicates that the practolol period preceded the H35/25 period.

and Fredholm 1976). On the other hand vasoconstriction induced by sympathetic nerve stimulation in canine subcutaneous adipose tissue was unaffected by propranolol. The presence of presynaptic β -adrenoceptors facilitating the release of noradrenaline from the sympathetic nerve terminals (see Langer 1977) may offer an explanation for these differences since any increased vasoconstrictor response due to blockade of the vascular β -adrenoceptors may be masked by the decreased noradrenaline release caused by a blockade of presynaptic β -adrenoceptors. It seems unlikely however that these two different actions of the β -blockade should exactly balance each other at all frequencies tested (0.5–8 Hz), especially since the presynaptic β -adrenoceptors seems to be of importance mainly at very low frequencies (see Langer 1977). A more likely explanation for the unaltered vasoconstrictor response to sympathetic nerve stimulation during β -adrenoceptor blockade is that noradrenaline released from nerve terminals cause α -adrenoceptor activation only. This would explain why sympathetic nerve stimulation invariably causes vasoconstriction in subcutaneous adipose tissue (Ngai *et al.* 1966). The present results are thus in good agreement with the hypothesis that vascular β -adrenoceptors in adipose tissue are humoral (non-innervated) receptors whereas the vascular α -adrenoceptors are innervated receptors (Rosell and Beltragi 1975, Beltragi and Rosell 1976).

During brief nerve stimulation there was little evidence for activation of vascular β -

TABLE I Effect of β -adrenoceptor blockade on a) escape of vasoconstriction during nerve stimulation at 4 Hz and b) peak vasoconstriction to i.a. injection of noradrenaline. The figures are given as mean \pm S.E. when $n > 3$

	Vasoconstriction % decrease in conductance		N	β -blocker
	Control	β -blockade		
a) Vasoconstriction at end of nerve stimulation for 30 min at 4 Hz	28 ± 8 25 ± 7 4 and 34	62 ± 4 47 ± 5 31 and 26	4 3	Propranolol Practolol H35/25
b) Peak vasoconstriction to noradrenaline 10^{-6} mol i.a.	50 ± 5 54 ± 3	62 ± 4 62 ± 2	5 4	Propranolol Practolol

Significant ($p < 0.05$) difference from corresponding control.

the vasoconstrictor escape during 30 min nerve stimulations at 4 Hz was only inhibited by practolol (Table I). Practolol but not 35/25 diminished the vasodilating effects of noradrenaline and nerve stimulation during α -adrenoceptor blockade whereas H35/25 but not practolol diminished the vasodilator response to salbutamol (Table II). Table III shows that practolol blocked the lipolytic responses to nerve stimulation and to noradrenaline to a greater extent than H35/25. However H35/25 was much more effective than practolol in blocking the lipolytic responses to salbutamol.

Discussion

In the present study it was confirmed that β -adrenoceptor blockade with propranolol potentiates the vasoconstriction induced by close i.a. injection of noradrenaline (Hjendahl

TABLE II Effect of β -adrenoceptor blockade on vasodilatation induced by a) sympathetic nerve stimulation after α -adrenoceptor blockade b) i.a. infusion of noradrenaline after α -adrenoceptor blockade and c) intraarterial infusion of salbutamol. The figures are then mean \pm S.E. changes during the last minute of infusion or nerve stimulation

		Concentration of SALB and NA	Vasodilatation % decrease in resistance	
a) Nerve stimulation α -blockade	Control	—	47 ± 9	3
	Practolol	—	28 ± 11	
	Control	—	$29/42$	
	H35/25	—	$36/38$	
b) NA-infusion α -blockade	Control	$7.2 \pm 1.6 \cdot 10^{-6}$ M	51 ± 9	1
	Practolol	$7.8 \pm 1.4 \cdot 10^{-6}$ M	29 ± 12	
	Control	$4.1/8.2 \cdot 10^{-6}$ M	$39/45$	
	H35/25	$6.2/9.8 \cdot 10^{-6}$ M	$42/51$	
c) SALB-infusion	Control	$1.3/1.4 \cdot 10^{-6}$ M	$47/53$	2
	Practolol	$1.6/2.1 \cdot 10^{-6}$ M	$52/63$	
	Control	$1.6 \pm 0.2 \cdot 10^{-6}$ M	46 ± 3	
	H35/25	$1.8 \pm 0.2 \cdot 10^{-6}$ M	$37 \pm 2^*$	

Significant ($p < 0.05$) difference from corresponding control.

TABLE II Effect of β -adrenoceptor blockade on lipolysis in 9 dogs. The net release of glycerol after staged treatments are given. When both β -blockers were used H35/25 was administered prior to practolol except in expt. 5 as indicated. SALB = 1 s. infusion of albutamol, N.S. = sympathetic nerve stimulation, NA = 1 s. injection or infusion of noradrenaline.

Expt. no.	Treatment	Net glycerol release (μ mol 100 g ⁻¹)		
		Control	H35/25	Practolol
1	SALB	42.8	—	35.9
2	SALB	5.2	0.3	3.8
3	SALB	70.7	15.4	37.4
	N.S.	62.5	90.2	10.8
4	SALB	13.3	2.2	6.2
	N.S.	24.8	17.1	0.4
5	SALB	69.9	33.0*	68.6
	N.S.	116.0	31.5*	27.6
	N.S.	79.6	—	23.7
6	N.S.	53.0	—	9.9
	NA	17.4	—	3.3
	NA	2.9	—	0
7	NA	5.5	—	0.5
	NA	40.2	—	14.9
8	NA (α -blockade)	3.8	—	1.2
	N.S. (α -blockade)	2.0	—	0.
9	NA (α -blockade)	7.3	9.8	0
	N.S. (α -blockade)	9.8	12.2	1.8

*Indicates that the practolol period preceded the H35/25 period.

and Fredholm 1976). On the other hand vasoconstriction induced by sympathetic nerve stimulation in canine subcutaneous adipose tissue was unaffected by propranolol. The presence of presynaptic β -adrenoceptors facilitating the release of noradrenaline from the sympathetic nerve terminals (see Langer 1977) may offer an explanation for these differences but any increased vasoconstrictor response due to blockade of the vascular β -adrenoceptors may be masked by the decreased noradrenaline release caused by a blockade of presynaptic β -adrenoceptors. It seems unlikely however that these two different actions of the β -blockade should exactly balance each other at all frequencies tested (0.5–8 Hz), especially since the presynaptic β -adrenoceptors seems to be of importance mainly at very low frequencies (see Langer 1977). A more likely explanation for the unaltered vasoconstrictor response to sympathetic nerve stimulation during β -adrenoceptor blockade is that noradrenaline released from nerve terminals cause α -adrenoceptor activation only. This would explain why sympathetic nerve stimulation invariably causes vasoconstriction in subcutaneous adipose tissue (Ngai *et al.* 1966). The present results are thus in good agreement with the hypothesis that vascular β -adrenoceptors in adipose tissue are humoral (non-innervated) receptors whereas the vascular α -adrenoceptors are innervated receptors (Rosell and Belfrage 1973, Belfrage and Rosell 1976).

During brief nerve stimulation there was little evidence for activation of vascular β -

adrenoceptors. However during prolonged nerve stimulation at 4 Hz there was a vasoconstrictor escape which could be diminished by propranolol. There are several alternative explanations to explain the mechanism behind this β -adrenoceptor mediated phenomenon. Firstly the slow onset of the escape might suggest that it is secondary to increased metabolism in the tissue. However there does not seem to be a simple relationship between the escape and lipolysis since the lipolysis at 1.5 Hz was almost as large as at 4 Hz. The combination of increased metabolism and a high degree of vasoconstriction may on the other hand be of importance since this may lead to accumulation of vasodilating products such as adenosine (Fredholm 1976) which may contribute to the escape phenomenon (Fredholm and Sollevik 1977). Secondly at higher frequencies, there might be a gradual overflow of noradrenaline from the nerve terminals at the adventitial-medial border to the humoral (noninnervated) β -adrenoceptors in the media of the blood vessels. At a lower frequency such as 1.5 Hz, the amount of noradrenaline reaching the vascular β -adrenoceptors could be too small to cause any significant activation of these receptors.

In the present study it was confirmed that sympathetic nerve stimulation after α -adrenoceptor blockade, invariably causes β -adrenoceptor mediated vasodilatation in adipose tissue (Ngai *et al* 1966). A possible explanation for these differences in β -adrenoceptor activation during normal condition and during α -adrenoceptor blockade may be a high availability of noradrenaline at β -adrenoceptors caused by the α -adrenoceptor blockade. This could be caused in two ways. Firstly presynaptic α -adrenoceptor blockade may cause increased noradrenaline release from the sympathetic nerve endings (see Langer 1977) and possibly the amounts reaching the β -adrenoceptors will be higher than during normal conditions. Secondly the lack of vasoconstriction caused by the postsynaptic α -adrenoceptor blockade may increase the noradrenaline outflow contributing to increased noradrenaline concentration (Rosell, Kopin and Axelrod 1964). Indeed, it has been found that α -adrenoceptor blockade increases the amounts of noradrenaline escaping to the venous effluent following sympathetic nerve stimulation in subcutaneous adipose tissue (Fredholm and Rosell 1970). Thus, although sympathetic nerve stimulation causes β -adrenoceptor mediated vasodilatation in adipose tissue during α -adrenoceptor blockade the present study suggests that during normal conditions there is no activation of vascular β -adrenoceptors of quantitative importance during sympathetic nerve stimulation, the vasoconstrictor escape being one possible exception.

In addition to a direct activation of vascular β -adrenoceptors catecholamines may also induce vasodilatation secondary to an increased metabolism in the surrounding tissue (see Lundholm 1966). In the present study it was found that the β -selective antagonist practolol blocked both lipolysis as well as four different manifestations of β -adrenoceptor mediated vasodilatation induced by noradrenaline or sympathetic nerve stimulation. These findings may support the view that the noradrenaline induced vasodilatation is secondary to lipolysis (Nielsen *et al* 1968). However there does not seem to be a causal relationship between those two parameters. Thus, nicotinic acid blocked lipolysis but not vasodilatation induced by L.v. noradrenaline (Mjøs and Akre 1971). Blockade of neuronal uptake of noradrenaline potentiated lipolysis but did not alter vasodilatation induced by L.v. injection of noradrenaline during α -adrenoceptor blockade (Rosell and Belfrage 1975, Belfrage and Rosell 1976).

more, acidosis blocked lipolysis but not vasodilatation induced by isoprenaline (Sjöblom and Fredholm 1976).

Present findings that practolol blocks both lipolysis and vasodilatation induced by noradrenaline may suggest that in adipose tissue both the β -adrenoceptors on the fat cells and the vascular β -adrenoceptors are of the β_1 -type. However in the present study both resistance and lipolysis induced by salbutamol were more effectively blocked by the β_2 -selective antagonist H15/25 than by practolol. Thus, both the vasodilating and the lipolytic responses to β_2 -selective agonists in adipose tissue are preferentially blocked by β_2 -selective antagonists whereas the same responses to β_1 -selective agonists are preferentially blocked by β_1 -selective antagonists. Carlsson *et al.* (1972) reported the same pattern of antagonist interactions in the cat heart and suggested that both β_1 - and β_2 -adrenoceptors are present in this tissue exerting the same response. Thus, both β_1 - and β_2 -adrenoceptors may be present both in the resistance vessels and on the fat cells in adipose tissue. Other factors such as the local distribution of these drugs in the tissue may also be of importance for the agonist-antagonist interaction patterns reported here for adipose tissue.

In conclusion the present study suggests that two factors may be of major importance for the vasodilating properties of intravascular noradrenaline in adipose tissue. Firstly the vascular β -adrenoceptors seem to be humoral (nonnervated) preferentially accessible to circulating noradrenaline. Neuroonally released noradrenaline may only during special circumstances be able to activate these receptors. Secondly the vascular β -adrenoceptors in adipose tissue seem mainly to be of the β_1 -type. Since vascular β -adrenoceptors in skeletal muscle are of the β_2 -type (Lands *et al.* 1967) the presence of β_1 -adrenoceptors in adipose tissue could offer one explanation why adipose tissue was the only tissue that responded with vasodilatation to intravenous infusion of noradrenaline (Hoffman and Forsyth 1973).

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Comparison of β -adrenoceptors mediating vasodilatation in canine subcutaneous adipose tissue and skeletal muscle

By

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Abstract

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and how changes in response to various drugs in simultaneously antoperfused canine subcutaneous adipose tissue and gracilis muscle were compared to study the vascular β -adrenoceptors. Compared to tyramine the β_2 -selective agonist salbutamol was 4-6 times more potent as vasodilator in the muscle as in adipose tissue. Furthermore two β_1 -selective agonists (Tazolol and H9042) caused vasodilatation in adipose tissue but not in the gracilis muscle. When given by close i.a. injection after β -adrenoceptor blockade, adrenaline is more potent vasoconstrictor than noradrenaline in both tissues. Before β -blockade, however noradrenaline was the more potent vasoconstrictor in the gracilis muscle whereas tyramine was more potent in adipose tissue. Intravenous infusion of adrenaline in doses causing vasodilation in the muscle caused vasoconstriction in adipose tissue whereas intravenous infusion of noradrenaline caused vasoconstriction in both tissues. The present findings suggest that the β -adrenoceptors mediating vasodilatation in skeletal muscle are mainly of the β_2 -type, whereas β_1 -adrenoceptors seem to predominate in subcutaneous adipose tissue. Since adrenaline is much more potent β_1 - than β_2 -agonist, our differences point to different roles of intravascular adrenaline in the two sites. In skeletal muscle adrenaline acts mainly as vasodilator whereas in subcutaneous adipose tissue it mainly acts as vasoconstrictor.

In contrast to other tissues (Hoffbrand and Forsyth 1973) subcutaneous adipose tissue may respond with β -adrenoceptor mediated vasodilatation during intravascular administration of noradrenaline (Nielsen *et al.* 1968 Ballard *et al.* 1971 Hoffbrand and Forsyth 1973). Sympathetic nerve stimulation, on the other hand, leads mainly to α -adrenoceptor activation (Belfrage 1978) and thus always causes vasoconstriction in adipose tissue (Ngai *et al.* 1966) as in other tissues. The vasodilating properties of intravascular noradrenaline in adipose tissue may have several explanations. Firstly vascular α - and β -adrenoceptors may have different distribution in relation to the sympathetic nerves—the α -adrenoceptors being increased receptors in close contact with nerve endings and the β -adrenoceptors being lateral (non-innervated) receptors situated closer to the lumen at some distance from the nerve terminals (Rosell and Belfrage 1975 Belfrage and Rosell 1976). Such an arrangement

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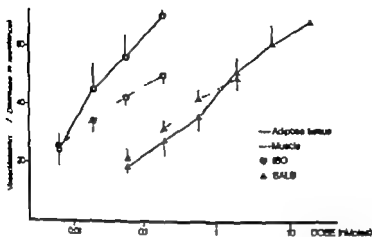


Fig. 1. Dose-response curves to β -isoprenaline (ISO) and salbutamol (SALB) in adipose tissue and in the skeletal muscle. Each symbol represents the mean \pm S.E. in four dogs. Salbutamol 20 nmol was tested in a dog only.

Results

Resting blood flows in the two tissues were calculated in 11 dogs and found to be $15.9 \text{ ml min}^{-1} 100 \text{ g tissue}^{-1}$ (range 6.3–26.2) in adipose tissue and $14.6 \text{ ml min}^{-1} 100 \text{ g tissue}^{-1}$ (12.0–16.5) in the gracilis muscle. Thus resting blood flows were similar in the two tissues although there was greater variation in adipose tissue.

In four dogs the vasodilating effects of isoprenaline and salbutamol were compared (Fig. 1). In the gracilis muscle the dose-response curves for the two compounds were parallel and salbutamol was about 12–16 times less potent than isoprenaline. The dose-ratio at the 50% level of 40% decrease in resistance was 14 ± 1.1 . In adipose tissue, on the other hand, the dose-response curve for isoprenaline was somewhat steeper than for salbutamol and dose-ratio calculations dependent on the size of the response. At 40 and 55% decrease in resistance the dose-ratios were 52 ± 8 and 93 ± 9 respectively. Both these two dose-ratios are significantly ($p < 0.05$) different from the dose-ratio in the skeletal muscle. Thus, in relation to isoprenaline, which is an agonist on both β_1 - and β_2 -adrenoceptors, the β_2 -selective agonist salbutamol was some 4–6 times less potent a vasodilator in adipose tissue compared with the gracilis muscle.

The two β_2 -selective agonists terbutaline (TTP) and H 80/62 induced dose dependent vasodilatation in adipose tissue (Fig. 2). Although these drugs were not always tested in the same preparations as isoprenaline it could be calculated that they were about 500 times less potent than isoprenaline by comparing the dose response curves of Fig. 1 and Fig. 2. Both terbutaline and H 80/62 failed to alter the blood flow in the gracilis muscle. The vasodilatations in adipose tissue induced by TTP and H 80/62 could be blocked by pretreatment with propranolol 500 μg i.a.

When given by close i.a. injection both noradrenaline and adrenaline caused dose dependent vasoconstriction in adipose tissue as well as in the gracilis muscle (Fig. 3). In control experiments adrenaline was the more potent vasoconstrictor in adipose tissue whereas noradren-

of vascular α and β -adrenoceptors has earlier been proposed for other tissues e.g. skeletal muscle (Glick *et al.* 1967). An additional explanation could be the existence of different types of vascular β -adrenoceptors in different tissues. Hjendahl and Fredholm (1976) suggested that adipose tissue in contrast to skeletal muscle may have a β -adrenergic vasodilator mechanism that is unusually sensitive to noradrenaline. Since noradrenaline is a somewhat specific β -agonist and vascular β -adrenoceptors in general are of the β_2 -type (Lands *et al.* 1967) a predominance of β -adrenoceptors in the resistance vessels of adipose tissue may help to explain the vasodilating properties of intravascular noradrenaline in this tissue. There is some experimental evidence that adipose tissue have vascular β -adrenoceptors of the β type (Belfrage 1978). To provide further experimental evidence for the existence of different types of vascular β -receptors I have in the present study made a direct comparison of vasodilatation mediated by β -adrenoceptors in s.c. adipose tissue and in skeletal muscle.

Methods

Female mongrel dogs weighing 10–22 kg were anesthetized with sodium pentobarbitone, 30 mg/kg supplemented as necessary. Tracheotomy was performed and the dogs were mechanically ventilated.

Subcutaneous adipose tissue weighing 43 g (range 28–55 g) in the inguinal region of 17 dogs was prepared according to the method of Rosell (1966). The adipose tissue was completely isolated from all surrounding tissues and supplied by one artery, one vein and a single nerve which was sectioned in all experiments. The collateral gracilis muscle weight 35 g (range 30–80 g) was completely isolated from surrounding tissue described by Renkin and Rosell (1962). This tissue was also denervated in all experiments.

Heparin, 2 500 LU/kg i.v. was administered to prevent clotting and the adipose tissue artery and artery to the gracilis muscle were cannulated and autoperfused from the ipsilateral femoral artery. Adipose tissue vein was cannulated in all experiments and the blood was returned to the femoral vein. The gracilis muscle vein(s) were not cannulated. Silicon filled drop recorders for blood flow registration were inserted in the arterial loops. Systemic blood pressure was measured in carotid artery and recorded together with blood flow on a Grass model 7B polygraph. Vasodilatation was calculated from the per cent decrease in resistance ($\text{mmHg } 100 \text{ g } \text{min}^{-1} \text{ ml}^{-1}$) and vasoconstriction from the per cent decrease in conductance ($\text{ml} \times \text{min}^{-1} 100 \text{ g}^{-1} \text{ mmHg}^{-1}$). Thus, both vasoconstrictions and vasodilatations are expressed in percentages between 0 and 100. Drugs were given simultaneously to both tissues by close i.a. bolus injection through side-arms in the arterial loops, proximal to the drop recorder. All drugs were freshly made from frozen stock solutions and diluted in saline containing 20 $\mu\text{g/ml}$ ascorbic acid. Injection volume was always 0.2 ml. Injection of this volume of the saline-ascorbic acid solution had no vascular effects. In experiments, where adrenaline and noradrenaline were injected, propranolol (500 μg i.a.) was administered to each tissue after the control dose response curves were determined and a second pair of dose response curves were then obtained. This dose of propranolol gives an almost complete blockade of β -adrenoceptors (Fredholm and Rosell 1968). In 5 of the experiments, adrenaline and noradrenaline were infused through brachial vein in different concentrations at a rate of 0.23 ml/min for 10–20 min. Infusion of the ascorbic acid solution at this rate had no effect on blood pressure or the adipose tissue and gracilis arterial blood flows.

In 4 additional experiments, two adipose tissue preparations were simultaneously perfused, one pretreated with propranolol (500 μg i.a.) and the other serving as control. The vascular as well as the lipolytic response to i.v. infusion of noradrenaline and adrenaline was measured. The release of glycerol was taken as an index of lipolytic activity and was measured according to the method of Laurell and Többling (1967). Venous blood samples were taken from the adipose tissue vein and arterial samples from the carotid artery. The glycerol release rate was calculated by multiplying the A-V difference by plasma flow in $\text{ml } \text{min}^{-1} 100 \text{ g}^{-1}$.

The following drugs were used: noradrenaline (L-arterenol HCl, Sigma), adrenaline (L-epinephrine bitartrate, Sigma), isoprenaline (L-isoprenaline bitartrate) salbutamol (salbutamol sulphate, Allen Hanbury), ITP (tizofol-HCl, Syntex), H 80/62 (\pm H 133/22, Hässle), propranolol (Inderal[®] JCI). Statistical evaluation was performed by using Student's *t*-test for paired observations.

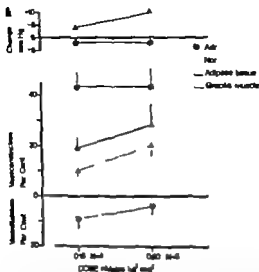


Fig. 4 Effect of i.v. infusion of adrenaline (Adr) and noradrenaline (Nor) in concomitantly perfused adipose tissue and skeletal muscle. The dose of $0.15 \text{ nmol kg}^{-1} \text{ min}^{-1}$ was tested in 4 dogs, whereas the (less) higher dose was tested in 3 dogs. Symbols represents the mean \pm S.E. In adipose tissue adrenaline was significantly ($P < 0.05$) more potent vasoconstrictor than noradrenaline at both doses.

adrenaline was infused at a rate of 0.15 or $0.60 \text{ nmol kg}^{-1} \text{ min}^{-1}$ there was a fall in the mean systemic blood pressure in all expts., as well as a decreased vascular resistance in the skeletal muscle (Fig. 4). However in adipose tissue the concomitant vascular reaction was vasoconstriction. Infusion of noradrenaline in the same doses (0.15 and $0.60 \text{ nmol kg}^{-1} \text{ min}^{-1}$) caused a small increase in systemic blood pressure and vasoconstriction in both

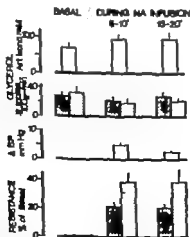


Fig. 5 Effect of i.v. infusion of $0.60 \text{ nmol kg}^{-1} \text{ min}^{-1}$ noradrenaline for 20 min on arterial glycerol release, glycerol release from two concomitantly perfused adipose tissue preparations, mean systemic blood pressure and vascular resistance in the adipose tissue preparations. One tissue was pretreated with the β -adrenoceptor blocking agent phentolamine $300 \mu\text{g i.v.}$ (Dotted bars \blacksquare) and the other served as control (white bars \square). Mean values \pm S.E.

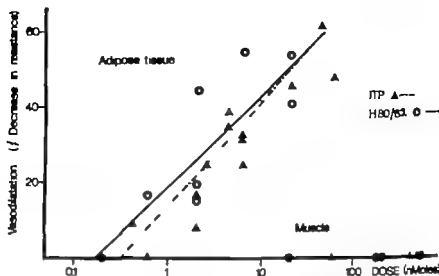


Fig. 2. Vasodilatation Induced by ITP (Tazolol) and H 80/62. Each symbol represents the effect of an i.a. injection. ITP was tested in 5 adipose tissue and 3 gracilis muscle preparations and H 80/62 in 4 adipose tissue and 3 gracilis muscle preparations. Regression lines of the vasodilatations in adipose are also shown.

aline was the more potent vasoconstrictor in the gracilis muscle. β -adrenoceptor blockade with propranolol (500 μ g i.a.) did not alter the relationship between the two amines in adipose tissue. However after propranolol adrenaline became more potent as a vasoconstrictor than noradrenaline also in the gracilis muscle.

Intravenous infusions of adrenaline and noradrenaline were performed in 5 dogs. When

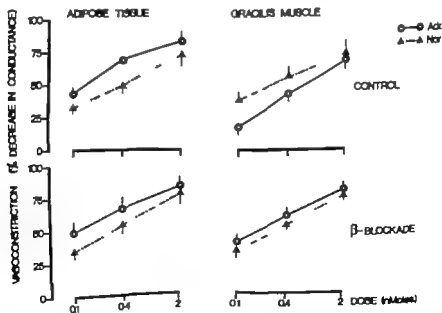


Fig. 3. Dose-response curves to i.a. adrenaline (Ad) and noradrenaline (Nor) in adipose tissue and in the gracilis muscle. Symbols represent the mean \pm S.E. in 5 dogs. During control conditions (on top) adrenaline was significantly ($p < 0.01$) more potent than noradrenaline in adipose tissue whereas noradrenaline was significantly ($p < 0.01$) more potent in the muscle. After β -adrenoceptor blockade (bottom) adrenaline was the most potent vasoconstrictor in both tissues ($p < 0.05$).

of great importance for their vascular responses. In the present expts., adrenaline more potent vasoconstrictor than noradrenaline in both tissues after β -adrenoceptor blockade. The present finding that adrenaline was a less potent vasoconstrictor than noradrenaline in the gracilis muscle but not in the adipose tissue, prior to β -blockade, could be explained by the predominance of adrenaline-sensitive β_2 -receptors in the muscle, and the α -adrenoceptors. During i.v. infusion of adrenaline the β_2 -receptor effects dominating in the gracilis muscle resulting in vasodilatation in this tissue. However, concomitantly perfused adipose tissue the vascular response was vasoconstriction. Moreover, before β -blockade adrenaline was a more potent vasoconstrictor in adipose tissue than noradrenaline both when the amines were given by close i.a. injection and when were infused i.v. Thus, the present study points to different roles of intravascular amines. In skeletal muscle it may mainly be a vasodilator whereas in adipose tissue it is a constrictor.

The present study has shown marked differences in β -adrenoceptors mediating vasodilation in adipose tissue and skeletal muscle. In the muscle only β_2 -adrenoceptors seem to be present whereas β_1 -adrenoceptors predominates in adipose tissue. The occurrence of the β_2 -adrenoceptors has been proposed also in other tissues such as the kidney (Taira, 1977) and the brain (Sercombe *et al.* 1977). The classification of vascular β -adrenoceptors into the β_1 -type (Lands 1967) may thus be far too generalizing. The present study indicates that the type of vascular β -adrenoceptor may be of great importance for the vascular response to endogenous catecholamines. This is further supported by the difference in the reaction in adipose tissue and in skeletal muscle to hemorrhagic shock in dogs in which blood flow in adipose is decreased to a much larger extent than in skeletal muscle (Rowell *et al.* 1973). Since both tissues were denervated, and since α -adrenoceptor blockade largely prevented the vasoconstriction it was suggested that increased levels of circulating catecholamines caused the severe vasoconstriction in adipose tissue. In skeletal muscle the α -adrenergic effects caused by increased blood levels of adrenaline may be masked by an concomitant β_2 -adrenoceptor activation. In adipose tissue, where vascular β_1 -adrenoceptors is of minor importance the increased levels of adrenaline seems to cause a severe, essentially unopposed vasoconstriction.

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adipose tissue and the gracilis muscle (Fig. 4). Compared to adrenaline, noradrenaline cause a much less pronounced vasoconstriction in adipose tissue. In adipose tissue the vasoconstrictions induced by 0.15 and 0.60 nmol \times kg⁻¹ min⁻¹ of both adrenaline and noradrenaline were sustained throughout the 15–20 min infusion.

In 4 expts. 2 adipose tissue preparations were perfused, one of which was treated with 500 μ g propranolol. As can be seen in Fig. 5 intravenous infusion of 0.60 nmol \times kg⁻¹ min⁻¹ of noradrenaline caused a slight increase in systemic blood pressure and an increase in the arterial concentration of glycerol. However, there was no increase in glycerol release from either the control preparation or that treated with propranolol. The vasoconstriction was significantly greater ($p < 0.05$) in the β -blocked tissue both at 5–10 min and at 15–20 min infusion.

Discussion

According to the classification of Lands *et al.* (1967) vascular β -adrenoceptors are of the β_2 -type. This seems to hold for the resistance vessels of the gracilis muscle of the dog, since the dose-ratio between the β_2 -selective agonist salbutamol and isoprenaline in the present study is in good agreement with what is usually found for such β_2 -mediated effects as vasodilatation in vascular beds of skeletal muscles (Cullum *et al.* 1969; Wasserman and Lev 1974; Taira *et al.* 1977) and bronchodilatation (Brittain *et al.* 1973; Cullum *et al.* 1969). In contrast, it has been suggested that the resistance vessels of dog subcutaneous adipose tissue contain mainly β -adrenoceptors, since a β - but not a β_2 -selective antagonist could block the vasodilating effects of noradrenaline (Belfrage 1978). In the present study 4–11 times higher doses of salbutamol in relation to isoprenaline was needed in adipose tissue in comparison with the gracilis muscle. Furthermore, tazolol and H 80/62 induced vasodilatation in adipose tissue but not in the gracilis muscle. Both tazolol (Lockwood and Lum 1974) and H 80/62 (Hedberg *et al.* 1977) has been shown to have β -selective agonistic properties. Thus, the present study supports the view that vascular β -adrenoceptors in adipose tissue, in contrast to skeletal muscle, are mainly of the β -type. However, vascular β_2 -adrenoceptors may also be present in adipose tissue since a β_2 - but not a β -selective antagonist could block vasodilatation induced by salbutamol (Belfrage 1978).

These tissue differences in vascular β -adrenoceptor types may be of considerable importance for the vascular responses to circulating adrenaline and noradrenaline since these catecholamines have different β - and β_2 -receptor agonist profiles. Noradrenaline is mainly a β -agonist (Lands *et al.* 1967). This may explain why adipose tissue in contrast to skeletal muscle responded with vasodilatation to i.v. noradrenaline (Hoffbrand and Forsyth 1973). Vasodilatation in adipose tissue to i.v. infusion of noradrenaline was not seen in the present study in contrast to others (Ballard 1973; Hjemsdahl and Fredholm 1974). Possibly these differences may be explained by higher resting blood flows and lower doses of noradrenaline in the present study. However, vascular β -adrenoceptor activation in adipose tissue could be demonstrated also in this study since β -adrenoceptor blockade potentiated the vasoconstriction induced by i.v. noradrenaline.

Adrenaline, in comparison with noradrenaline, is a much more potent β_2 -agonist and weaker β_1 -agonist (Lands 1967). The α -adrenoceptor agonistic properties of the two amines

The *in vitro* transport of ^{14}C - α -aminoisobutyric acid into mouse blastocysts during activation for implantation

By

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Abstract

FEL, L., B. EDENBERG, O. NILSSON and G. RÖNQVIST. The *in vitro* transport of ^{14}C - α -aminoisobutyric acid into mouse blastocysts during activation for implantation. *Acta physiol. scand.* 1978. 102. 477-483.

The transport of ^{14}C -AIB (^{14}C - α -aminoisobutyric acid) into mouse blastocysts was studied during *in vitro* activation. Mice, kept in experimentally delayed implantation, were given oestrogen to initiate and then injections of ^{14}C -AIB 1, 2, 4 and 8 h after the oestrogen. After *in vitro* activation for 1/3 or 4 h the labeled amino acid the blastocysts were flushed out of the uterus. A distinct uptake of ^{14}C -AIB occurred in the blastocysts 8 h (the highest uptake) and 12 h after activation, provided that the *in vivo* incubation time was 4 h. At these times the blastocysts are free in the uterine lumen and consequently there is transport of ^{14}C -AIB from the epithelium via the uterine lumen into the blastocysts. This uptake indicates that amino acids transported by the system are important nutrients during early activation. The uptake and retention of ^{14}C -AIB in the uterus was highest at 4 and 8 h after the induction of implantation. The highest uptake was observed when the labeled amino acid was given at 8 h while the longest retention time occurred when ^{14}C -AIB was given at 4 h. The transport rate between the blastocysts and the uterine lumen is not maintained constant, indicating that the transport rates for ^{14}C -AIB transport are different for the uterus and the blastocysts. The AIB transport into uterine tissue precedes that into the blastocysts. The AIB transport into blastocysts is maintained as long as they have negative surface charge.

variations in nutrient uptake activity that accompany changes in cellular proliferation have been implicated in models of cell cycle control involving regulation of surface charge functions (Hodley 1972, Pardoll 1974, Cecchini *et al.* 1976). However studies of variations of transport rate of nutrients such as certain amino acids into intact cells have been difficult to interpret due to the complexity of the system. Thus, it has been dif-

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TABLE I. Radioactivity of samples of buffer used for flushing and washing blastocyst in one representative experiment.

	Counts/10 min. \pm sg
Flushing rat decidua buffer including uterine and g. uterine secretions	15
1st washing buffer	12
2nd washing buffer	10
3rd buffer	10

Electron microscopy preparation. Electron microscopy of the uterine epithelium was performed on uteri obtained from mice which had been influenced by estrogen for 8 h and by AIB for 4 h, and then fixed for blastocysts according to the standard procedure used in the experiments. Uterine horns from 3 mice were carefully fixed by an extraabdominal injection of a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The specimens were dehydrated in acetone and embedded in Vestopal. One section from each animal is examined.

Blastocysts for electron microscopy were obtained from 2 mice which had been given estrogen 8 h and AIB 4 h before the preparation. The blastocysts are flushed and incubated according to the procedure usually used. They were then fixed in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, dehydrated in ethanol, and embedded in Epon. Two blastocysts from each animal were examined.

Results

The blastocysts demonstrated a conventional appearance without any sign of damage to the trophectoderm layer when inspected with the transmission electron microscope (Fig. 1). The structure of the uterine epithelium after the flushing was also normal (Fig. 2) indicating that no substantial amount of cytoplasmic content could have contaminated the flushing fluid. Both tissues were examined 8 h after the induction of implantation, with an *in vivo* incubation time of AIB for 4 h.

Further, only the buffer used for flushing out the blastocysts contained measurable radioactivity while the final washing had background counting (Table I).

The *in vivo* transport of ^{14}C -AIB into the blastocysts is clearly seen 8 h after the induction of implantation while only a tiny transport occurs 12 h after the induction provided that the *in vivo* incubation time was 4 h (Table II). No transport at all was measurable neither when the labeled amino acid was given concomitantly with the estrogen nor at any time long activation for implantation with an *in vivo* incubation time for only 1/3 h of ^{14}C AIB.

TABLE II. The uptake of ^{14}C -AIB in blastocysts *in vivo*.

Number of blastocysts	Estrogen activation time, hours	^{14}C AIB incubation time, hours	Radioactivity of the blastocysts, counts/10 min
3	4	1/3	0
3	4	4	8
3	8	1/3	0
3	8	4	80
3	8	4	45
3	8	4	60
3	12	4	10
3	12	4	15

difficult to distinguish unambiguously between effects of intracellular metabolic changes, changes associated with the plasma membrane and effects of surface area on transport rates. Attempts have been made to separate uptake from subsequent metabolism by the use of non-metabolizable analogues, such as α -aminoisobutyric acid, which is transported by the system A as defined by Christensen (1975).

A favoured *in vivo* transport of ^{14}C AIB was found to occur into mouse uterine tissue by estrogen-injected animals in delayed implantation provided that the estrogen injection preceded that of the ^{14}C AIB by 4 to 8 h (Lindqvist *et al.* 1977). Thus, during activation of implantation the first 8 h are a period when some amino acids are supplied from the blood of the mice into the uterine tissue at a high degree.

Since induction of implantation by estrogen administration is associated with an activation of the metabolism of the blastocyst (McLaren 1973) the uterine response could indicate an increased capacity to supply the blastocyst with amino acids. Therefore, the aim of the present studies was to examine whether also the blastocysts demonstrated a preferential transport of ^{14}C AIB *in vivo* during activation for implantation.

Material and methods

^4C - α -aminoisobutyric acid (1 ^{14}C AIB), Protosol, and Aquasol were purchased from NEN Chemicals GmbH, Dreieichenbahn, W. Germany and α -aminoisobutyric acid from Calbiochem, San Diego, USA. Depo-Provera was purchased from Upjohn a.s., Puurs, Belgium and estradiol-17- β from AB Leo, Helsingborg, Sweden. All chemicals used were analytical grade. Radioactivity was measured in Nuclear Chicago Uniflux II Liquid Scintillation Counter.

Adult white female mice (N M R.1. Antikemex, Stockholm, Sweden) were mated and the day the sperm plug was found was recorded as day 1. The mice were sprayed on day 3 and given a first dose of Depo-Provera (1 mg/0.05 ml) subcutaneously the same day to keep the blastocysts in delayed implantation (Humphrey 1967 Bergström 1977). A second dose was administered on day 10 or 11 and the animals were taken for experiments on day 11–13.

In vivo incubation procedure. Implantation was initiated by subcutaneous injection of 0.1 μg estradiol-17- β in 0.1 ml propylene glycol either concomitantly with or at different times prior to the administration of ^{14}C -AIB. The labeled amino acid (4.25 μCi ^{14}C AIB in 0.1 ml of a 40 mM AIB-solution, made isotonic with NaCl) was given in the tail vein. The mice were anesthetized by Nembutal and the uterus was removed within 5 min. The blastocysts were flushed out of the uterine horns with an ice-cold Krebs-Ringer choline-bicarbonate buffer (all sodium ions were stoichiometrically replaced by choline ions), freshly bubbled with a gas mixture of 93.5% O_2 and 6.5% CO_2 to give final pH of 7.4. The buffer also contained 100 mg bovine albumin/30 ml buffer to prevent stickiness of the blastocysts and to stabilize the blastocyst membranes (*cf.* Kromphardt 1968). The buffer was ice-cold and N_2 -free in order to immobilize the AIB-carrier for transport.

The blastocysts were washed in the same ice-cold buffer as was used for the flushing out, transferred to fresh buffer, counted and collected using siliconized glass capillary and transferred to a weighed scintillation vial together with a small amount of buffer. During this procedure the blastocysts displayed an unchanged appearance as observed in the preparation microscope. Buffer plus blastocysts were then weighed. Samples of the flushing out buffer, the later washings and of pure buffer were also weighed in vials (Table I). All samples were dissolved in 1 ml of Protosol, 50 μl conc. acetic acid and 10 ml of Aquasol were added and the radioactivity was measured. Duration of blastocyst activation, ^{14}C -AIB incubation, and the number of blastocysts in each experiment are shown in Table II.

Uterine tissue samples for the retention experiments were treated as described before (Lindqvist *et al.* 1977), that is the uterine horns were removed from the anesthetized animals, trimmed and washed in ice-cold buffer, lightly blotted on filter paper, weighed in scintillation vials and dissolved in Protosol overnight. The solution was mixed with conc. acetic acid and Aquasol and the radioactivity measured. Each retention value is the mean of the values derived from 3–6 animals (Fig. 3).



Fig. 1. Mouse epithelium from specimen obtained from mouse given estrogen 8 h and AIB 4 h prior to fixation and then fixed for blastocysts according to the procedure used in the experiments. The micrograph is representative of this type of epithelium demonstrating, among other things, apical protrusions and conventional outlines of the nuclei. The circular granules are lipid granules. $\times 2,700$.

transported onto the blastocyst surface nor had diffused into the blastocoelic fluid of the blastocysts. Considering that the second washing fluid never contained any measurable radioactivity, no leakage of labeled amino acid occurred from the blastocysts into the buffer. The measured radioactivity of the isolated and washed blastocysts represented labeled amino acid taken up by the blastocysts.

The transport ratio between blastocysts and uterine tissue were different for different times indicating an autonomous transport of ^{14}C AIB for the blastocysts as compared to the uterus. The retention curves for labeled amino acid in uterine tissue displayed a turnover when ^{14}C AIB was administered later during the activation for implanta-

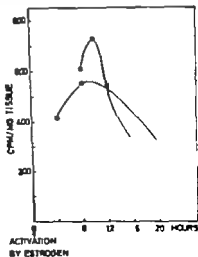


Fig. 2. Retention of ^{14}C -AIB in mouse uterine tissue at different times after induction of implantation. ●—●—● ^{14}C -AIB measured 4 h after induction, ■—■—■ ^{14}C -AIB measured 8 h after induction. Max. stand. dev. ± 50 cpm.



Fig. 1 Part of a blastocyst obtained from a mouse given estrogen 8 h and AIB 4 h before the preparation and incubated according to the procedure used in the expts. The ultrastructure shows a representative of this type of blastocysts demonstrating unchanged trophoblastic cells with normal-looking mitochondria. $\times 9500$.

The initial preferential uptake of ^{14}C -AIB in the uterus was higher at 8 h than at 4 h after the induction of implantation. The retention time was shorter when ^{14}C -AIB was given at 8 h than when given at 4 h (Fig. 3).

The transport ratio between blastocysts and uterine tissue

$$\left(\frac{\text{cpm/total number of blastocysts in each expt.}}{\text{cpm/mg uterine tissue}} \right)$$

was not maintained constant but grew from 0 at an activation time of 4 h to a maximum value of 0.011 at 8 h activation and then decreased to 0.003 after 12 h activation with an *in vivo* incubation time of 4 h in all expts. The discrepancy between uterine tissue and blastocysts at ratio 0 is further strengthened by the fact that the transport rate of ^{14}C -AIB into uterine tissue, is considerable already 1/3 h after the induction (Lindqvist *et al.* 1977) while still 4 h after the induction the transport into the blastocysts is not measurable (Table II).

Discussion

The blastocysts *in situ* displayed indeed under certain conditions an accumulation of ^{14}C -AIB administered *in vivo*. The peak value was found at 8 h after the injection of estrogen. At this time the blastocysts were not in direct contact with the uterine wall but lay in the uterine secretion (Nilsson 1974). Since the amount of radioactive amino acid in the uterine secretions surrounding the blastocysts was in excess of that taken up by the blastocysts and since the morphology of the tissues was normal, an adsorption of uterine epithelial fragments containing labeled amino acid onto the blastocysts hardly seems probable. This conclusion is strengthened by the expts. with the shorter *in vivo* incubation for only 1/3 h of ^{14}C -AIB. The radioactivity then was found in the flushing fluid while the washed blastocysts never contained any radioactivity. This indicates that AIB artifactually neither had



Fig. 1. Electron micrograph of uterine epithelium from a mouse specimen obtained from a mouse given estrogen 8 h and AIB 4 h in preparation and then flushed for blastocysts according to the procedures used in the experiments. The area shown is representative of this type of epithelium demonstrating, among other things, an apical epithelium lining with apical protrusions and conventional outlines of the nuclei. The circular body-stained areas are lipid granules. $\times 2700$.

adsorbed onto the blastocyst surface nor had diffused into the blastocoelic fluid of the blastocysts. Considering that the second washing fluid never contained any measurable radioactivity, no leakage of labeled amino acid occurred from the blastocysts into the buffer. The measured radioactivity of the isolated and washed blastocysts represented labeled amino acid taken up by the blastocysts.

The transport ratio between blastocysts and uterine tissue were different for different times indicating an autonomous transport of ^{14}C -AIB for the blastocysts as compared to the uterus. The retention curves for labeled amino acid in uterine tissue displayed a turnover when ^{14}C AIB was administered later during the activation for implanta-

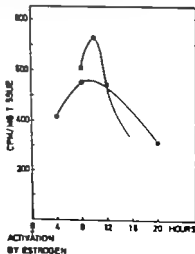


Fig. 2. Retention of ^{14}C -AIB in mouse uterine tissue at different times after induction of implantation. ●—●—● ^{14}C -AIB administered 4 h after induction, □—□—□ ^{14}C -AIB administered 8 h after induction. Max. stand. dev. ± 50 cpm.

tion. This shorter retention time, when the radioactive amino acid was administered late during activation for implantation, explained the lower content of radioactivity in the blastocysts at 12 h after induction. Since the 8 h peak appeared at a time when the blastocysts are not in direct contact with the uterine wall it is reasonable to conclude that a vectorial transport of ^3H AIB took place from the uterine epithelium via the uterine secretion into the blastocysts, and that no simple diffusion mechanism exists between the uterine epithelium and the blastocysts. Further since the transport ratio between the uterine tissue and the blastocysts is not maintained constant it is concluded that the metabolic rates for amino acid transport are different for the uterus and the blastocysts. Reasonably the AIB transport into uterine tissue precedes that into the blastocysts.

The space between the activated blastocysts and the uterine epithelium is filled with uterine secretion. This secretion contains the labeled AIB as well as other amino acids (Pratt 1977). Considering that *in vitro* expts. have shown the importance of some amino acids for the normal development of the blastocyst (Naesslund 1976), the system A and/or system L amino acids when reacting with their receptors at the blastocyst surface, can be working as trigger substances for blastocyst activation.

A parallelism exists between the *in vivo* transport of ^3H AIB into blastocysts and the surface charge of the blastocysts, since an uptake occurs as long as the blastocysts retain their negative surface charge (Nilsson *et al.* 1975). Simultaneously with the loss of negative surface charge the *in vivo* transport of ^3H AIB into the blastocysts is also diminishing. This is interesting to note taking into account similar phenomena displayed by malignant cells, studied *in vitro*. For instance, the negative charge on animal cell surfaces appears to be greater on several types of transformed cells as compared with their normal counterparts (Wallach and Eylar 1961; Forrester *et al.* 1964; Kraemer 1966), and the ^3H AIB transport capacity of neoplastically transformed glioma cells was several times higher than that of glia cells (Ronquist *et al.* 1976). Thus the activation of the mouse blastocyst has some similarities with the transformation of some strains of tissue culture cells. Often a direct relationship exists between the amount of sialic acid released by e.g. neuraminidase treatment and reduction of cellular electrophoretic mobility due to loss of negative charge at the cell surface (Weiss and Mayhew 1966). Since the removal of surface membrane sialic acid of HeLa cells resulted in a sharp decrease in ^3H AIB transport into these cells (Brown and Michael 1969) there might be a correlation between surface content of sialic acid and AIB transport. Hence the ^3H -AIB transport into blastocysts studied *in vivo* seems to have some characteristics as regards amino acid transport by the system A in common with other cell types studied *in vitro*. However the blastocysts are able to control the uptake process displaying an AIB transport only as long as the negative charge is maintained (Nilsson *et al.* 1975), while tumor cells seem not to be able to change their negative surface charge and consequently not the system A transport capacity.

The presence of sodium ions is a necessary prerequisite for an active transport of ^3H AIB into the blastocyst. An increased content of Na⁺ in the endometrium of rats 18 h after induction of implantation was reported by Setty *et al.* (1973). It was also claimed by Borland *et al.* (1977) that the Na⁺-movement into the blastocoel of mice might be energetically favoured at the approximate time for blastocyst activation. The sodium accumulation in-

the blastocysts support the view that the blastocysts themselves contain system A carriers with a co-transport of Na⁺ together with the ¹⁴C AIB transport. Work on isolated mouse blastocysts for studies of the carrier function is under progress.

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Influence of indomethacin and of prostaglandin E_1 on total and regional blood flow in man

By

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Abstract

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The central and regional circulatory effects in man of the prostaglandin synthetase inhibitor indomethacin and of prostaglandin E_1 (PGE_1) were studied. Systemic blood pressure, cardiac output, and renal and splanchnic blood flow were measured at rest, following infusion of indomethacin (50 mg i.v.), and during infusion of PGE_1 (4-8 μ g min⁻¹ l.) after the administration of indomethacin. An increase in the total, systemic resistance (+20%), as well as in the renal (+30%) and splanchnic (+16%) vascular resistance developed rapidly following the administration of indomethacin. Infusion of PGE_1 completely restored the resistance in the renal and splanchnic regions, and in addition markedly increased the blood flow in non-visceral tissues. We suggest that the circulatory effects by indomethacin are exerted via the drug's inhibitory effect on prostaglandin synthetase in the vessel walls, and that vasodilating products of PG synthetase affect the regional blood flow distribution in man.

It has been known for at least forty years that some of the prostaglandins decrease the systemic blood pressure (Euler 1936). This pharmacological action by PGs, well documented in isolated preparations as well as in intact animals of different species, has not yet been convincingly shown to have a physiological equivalent. Apparently the lack of receptor inhibitors for the action of PGs on vascular smooth muscle has hampered the demonstration of possible effects of endogenously formed PG in the circulatory system. With the discovery by Vane (1971) that certain anti-inflammatory drugs inhibit the bioformation of PGs, a tool was provided for demonstrating the contribution of endogenous PGs in physiological mechanisms. Thus, following infusion of the PG synthetase inhibitor indomethacin, an increase in the systemic blood pressure was observed in rabbits (Ånggård and Larsson 1973) as well as in dogs (Lonigro *et al.* 1973), possibly due to an inhibited formation of vasodilating PGs in the vascular bed of the animal. The observation was later confirmed in man (Wengmalm 1974, Patak *et al.* 1975), inasmuch as infusion of indomethacin i.v. was followed by a decrease in cardiac output and a parallel increase in the systemic blood pressure. These data thus indicated that indomethacin increases the systemic vascular resistance in man.

In the present study the possible influence of vasodilating PGs in the regional blood flow distribution in man has been studied further. First, the regional blood flow changes following administration of indomethacin were measured, and second, the ability of PGE₂ to restore these blood flow changes caused by indomethacin, was investigated.

Methods

The study was performed with the permission of the Ethical Committee at the Karolinska Institute. 19 healthy men, aged 23-41 years, participated. They were all informed of the nature, purpose and possible risks of the investigation before giving their voluntary consent to take part in the study. All investigations were performed in the morning after an over-night fast.

In 10 of the volunteers, the following experimental procedure was performed. A 3-way-Gassz catheter (no. 7) was inserted percutaneously into a selected cubital vein or into the right femoral vein and passed under fluoroscopic control in the pulmonary artery. T. Courmand catheters (no. 7 or 8) were inserted similarly and passed to the right-sided hepatic vein and to the right or left renal vein, respectively. T. short catheters were inserted into superficial forearm vein and brachial artery respectively. Pressure recordings were performed via pressure transducer (Elema EMT 35) on photostimulograph (Abeam Clinacord). Expired air was collected in Douglas bags and analyzed by the Scholander microtechnique. Blood samples were drawn from the brachial and pulmonary arteries for determination of oxygen content. The cardiac output (C.O.) was measured using the direct Fick method.

A solution of indocyanine green dye (Cardio-Green HW & D) and paraaminosalicylate (Sodium aminosalicylate, MSD) was infused into the short venous forearm catheter. Simultaneous arterial and hepatic and renal venous blood samples were drawn at regular intervals for determination of dye and PAH concentrations. Splanchnic and renal blood flows were estimated using the constant infusion technique (Bradley 1946).

Indomethacin (MSD, purity > 99%) was dissolved in ethanol at a concentration of 5 µg/ml and passed through sterile filter. Immediately prior to the infusion, 10 ml of this solution was added to 25 ml of 0.25 M phosphate buffer pH 7.5. The solution was infused at a rate of 1-2 ml/min via the 3-way-Gassz catheter. Sterile prostaglandin E₂ as obtained from Astra Läkemedel AB. It was dissolved in ethanol at a concentration of 100 µg/ml. Immediately prior to the infusion it was diluted in saline to a final concentration of 4 µg/ml. This solution was infused via the 3-way-Gassz catheter at a rate of 1 or 2 ml/min.

During the experiments, the subjects rested comfortably in the supine position. After the first 35 min of the PAH infusion, during which the plasma levels of these indicators stabilized, blood pressure and total and regional flow measurements were performed twice with a 25 min interval. Subsequently indomethacin was infused as described above. After the end of the indomethacin infusion, blood pressure and flow measurements were repeated twice with a 25 min interval. Thereafter PGE₂ infusion was started at a rate of 4 µg/min. In 4 subjects, who did not display any decrease in blood pressure after 5 min of PGE₂ infusion at 4 µg/min, the infusion rate was decreased to 2 µg/min. The total time of infusion of PGE₂ was 25-40 min. Total and regional flow measurements were started after 15 min of PGE₂ infusion. The measurements are reported as close as possible before the discontinuation of the infusion of PGE₂.

The remaining 9 subjects served as controls and were subjected to a simplified experimental procedure, in which only the cardiac output and the systemic blood pressure were estimated, at rest and following infusion of indomethacin solution.

Catecholamine Non-splanchnic blood flow was estimated by subtracting the actual figures on renal and splanchnic flow from the cardiac output. Data in the text and figures are expressed as mean \pm S.E. Figures in brackets indicate the number of expts. Student's t-test for paired differences has been used when applicable.

Results

Central and regional blood flow before drug administration

At supine rest before the infusion of drugs, all circulatory and respiratory parameters recorded were well within the normal limits. The cardiac output (6.5 l/min) was distributed in the tissues as follows. $26 \pm 2\%$ (9) perfused the kidneys, $19 \pm 1\%$ (10) passed through the

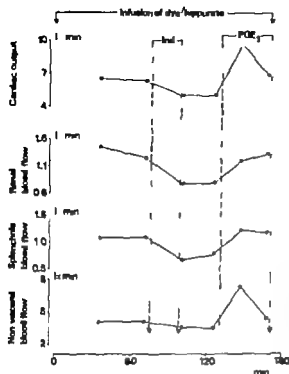


Fig. 1 Typical experiment demonstrating the effect of indomethacin (IND 50 mg infused i.v. during 20 min) and of prostaglandin E_2 (PGE 160 μ g infused i.v. during 40 min) on cardiac output, and on renal, splanchnic, and non-visceral blood flow in a healthy male volunteer aged 31.

splanchnic region, and the remaining $55 \pm 3\%$ (9) was thus directed through the non-visceral tissues.

b Effect of indomethacin on the central and regional circulation (Fig. 1 2)

Within 2–3 min of starting the infusion of indomethacin, increases in the systolic and diastolic blood pressures were observed. The elevation of the systemic blood pressure was fully developed within 10–15 min of infusion and persisted until PGE was infused (see below). Since cardiac output was insignificantly affected the increased systemic blood pressure must have been due to an increased overall vascular resistance in the systemic circulation. In fact the systemic vascular resistance was increased significantly by $20 \pm 8\%$ (10). However the regional resistances were not equally affected by indomethacin. There were significant increases obtained in the renal vascular resistance (by $30 \pm 9\%$, $n = 9$) and in the splanchnic resistance (by $16 \pm 6\%$, $n = 10$), while the vascular resistance in the non-visceral organs was insignificantly affected. Oxygen uptake and R value did not differ from resting values, indicating that the drug did not induce any metabolic effects.

In the 9 subjects infused with indomethacin solvent only no changes were observed in systemic vascular resistance (93 ± 8 kPa 1 s before and 97 ± 7 kPa 1 s after the infusion).

In no case did infusion of indomethacin or solvent elicit any kind of discomfort in the subjects. No side effects of the drug were observed at reexamination 1–2 weeks after the investigation.

c Effect of PGE on the central and regional circulation (Fig. 1 3)

During continuous infusion of PGE at a rate of 4–8 μ g/kg b.w./min after indomethacin hemodynamic circulation appeared, with increased cardiac output and decreased A–V O₂

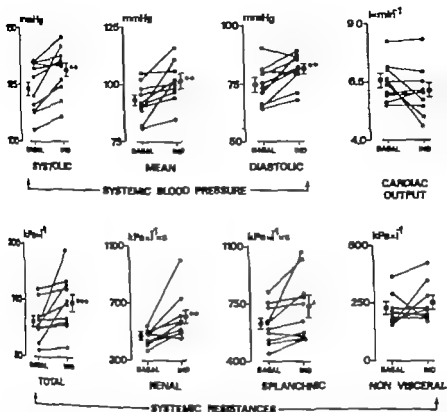


Fig. 2. Systemic blood pressure, cardiac output, and regional vascular resistances at rest (BASAL), and following administration of 50 mg of indomethacin (IND.). The smaller symbols represent the individual decreases and the larger symbols indicate means \pm S.E. in the respective groups. ** or *** indicates that the mean differs significantly ($P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively) from the corresponding value before indomethacin.

difference (from 45 ± 2 ml/l before to 33 ± 2 ml/l during the infusion, $n = 10$). The systemic blood pressure returned to the level observed before the infusion of indomethacin, and consequently the increased cardiac output was paralleled by a decreased overall systemic vascular resistance. The estimated systemic resistance was decreased by $23 \pm 6\%$ (10) con-

Table 1. Regional blood flow before indomethacin and during an i.v. infusion of PGE₂ after administration of indomethacin (50 mg). The values are presented as mean \pm S.E. Figures within brackets indicate number of experiments.

	Renal blood flow ml min ⁻¹	Splanchnic blood flow ml min ⁻¹	Non-visceral blood flow ml min ⁻¹
IND	1610 ± 70 (9)	1220 ± 70 (10)	3480 ± 250 (9)
Dose of PGE ₂ , 0.4 μ g min ⁻¹	1640 ± 100 (10)	1210 ± 70 (10)	3830 ± 600 (10)

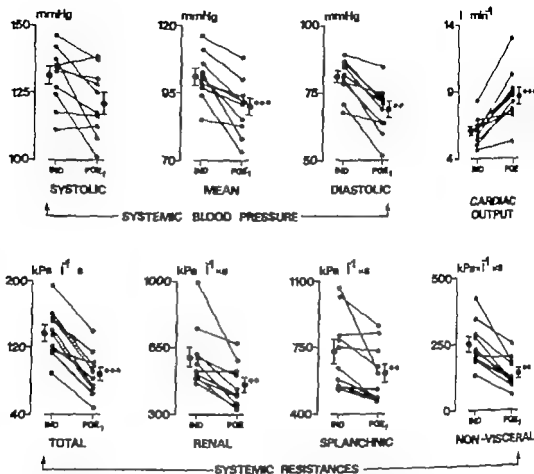


Fig. 3 Systemic blood pressure, cardiac output, and regional vascular resistances 25 min after administration of indomethacin (IND 50 mg), and during infusion of prostaglandin E₂ (PGE₂ 4 µg/min). The small symbols represent the individual observations and the larger symbols indicate mean \pm S.E. in the respective groups. * or ** indicates that the mean differs significantly ($P < 0.01$ or $P < 0.001$ respectively) from the corresponding figure before PGE₂.

pared with before indomethacin. The increased cardiac output was not distributed uniformly to the different organs. In the kidneys and the splanchnic region, the flow was increased no more than to the resting level observed before indomethacin (Table I). Since the cardiac output was augmented beyond this level, the non-visceral tissues must have received considerably higher flow during PGE₂ infusion than before infusion of indomethacin (Table I). The estimated local vascular resistances verify the uneven increase in flow during PGE₂ infusion: the resistance in the non-visceral tissues was decreased by $29 \pm 12\%$ (9), while the renal and splanchnic vascular resistances were unchanged compared to the values before indomethacin. No changes in the oxygen uptake or the R value were observed indicating that PGE₂ in the present dose lacks general metabolic effects.

In one subject, infusion of PGE₂ (8 µg/min kg b.w.) elicited abdominal pains, which disappeared following immediate discontinuation of the infusion. The remaining subjects

Discussion

Infusion of indomethacin in the current experiments induced distinct circulatory effects. The unchanged circulation following the infusion of drug solvent only revealed that indomethacin was the active principle and that the solvent ethanol was in itself without effect. General indomethacin elicited an increase in blood pressure parallel with an unchanged or slightly decreased cardiac output. These changes in the direction of a more hypokinetic circulation indicate that the primary effect of the drug was to increase the peripheral resistance. The increase in resistance was more prominent in the kidneys than in the remaining part of the systemic circulation. The varying effect of indomethacin in the different regions suggests that the drug effect was selective and not due to a general (central) vasoconstrictive action in the systemic resistance bed. It therefore appears that indomethacin acted peripherally possibly by interfering with local vasoregulatory mechanisms. Indomethacin is a well-known inhibitor of the biosynthesis of prostaglandins (Vane 1971). Since many PGs, e.g. those of the A, E, G and I series) are vasodilators, the currently observed systemic vasoconstriction caused by a PG synthetase inhibitor may have been due to the decrease of endogenous PGs from certain parts of the systemic resistance vascular bed. Indomethacin has recently been shown to inhibit PG synthesis in man considerably at a plasma drug concentration of 0.1 µg/ml (Rane *et al.* 1977). Assuming an indomethacin *ED*₅₀ of 30 l in the subjects in the present study a drug concentration of 0.1 µg/ml would be reached after 2-3 min of infusion. Thus, the currently observed rapid onset (within 2-3 min) of the blood pressure increasing effect of indomethacin seems plausible with respect to inhibition of PG synthesis.

It has earlier been shown in dogs (Lonigro *et al.* 1973) as well as in rabbits (Collins-Chourio *et al.* 1975), that the renal vascular tone is counteracted by intra-renal formed PGs and that inhibition of their synthesis is followed by an increase in the systemic blood pressure. In current results, which clearly demonstrate an increased vascular resistance following infusion of indomethacin, extend these observations to the supine, un-anesthetized man. However the currently observed effect of indomethacin on the human renal blood flow was considerably less than that obtained in the above-mentioned dog study (Lonigro *et al.* 1973). The observed effect of indomethacin in the splanchnic region was smaller than the corresponding drug action in the kidneys. Nevertheless it deserves attention since an effect of indomethacin, which may reflect synthesis of endogenous vasodilating PGs in the splanchnic vascular bed, has not been observed before. The resistance in the non-visceral tissues, which usually represent skin and skeletal muscle, was not affected in the present study. This is in contrast with earlier observations, demonstrating an unchanged resting skeletal muscle blood flow following indomethacin in dogs (Herbaczynska-Cedro *et al.* 1973) and in humans (Elborn and Wernholm 1976).

Infusion of PGE₁ completely reversed the resistance effect of indomethacin in the kidneys and the splanchnic region, and in addition markedly decreased the non-visceral resistance. The resistance vessels in the various regions were thus all relaxed when exposed to PGE₁, to a different degree. This indicates that the resistance changes caused by the preceding infusion of indomethacin were not only—if at all—induced by the withdrawal of endo-

generously produced PGs of the E series from the vascular bed. Other vasodilating PGs, like those at the A, G and I series, the synthesis of which is also inhibited by indomethacin, may have contributed to the lower "pre-indomethacin" resistance as well. Of special interest in this connection is the newly discovered PGI (Gryglewski *et al.* 1976, Bunting *et al.* 1976) which is a potent vasodilator and seems to be selectively formed in vessel walls. Armstrong *et al.* (1977), infusing PGI₁ in the anesthetized open-chest dog demonstrated a decrease in systemic blood pressure and total peripheral resistance. Analysis of the regional effects of PGI₁ have, however not been reported hitherto.

Interestingly the vascular bed that displayed the highest sensitivity to PGE₂, i.e. the non-visceral tissues, was insignificantly affected by indomethacin. Although the lack of effect of indomethacin on the non-visceral resistance implies that no production of vasodilating PGs was going on in these vessels during the present experimental conditions, the high vascular sensitivity to PGE₂ in this region may still be of significance. Much attention has recently focused on the possibility that endogenous vasodilating PGs may take part in the development of reactive hyperemia in coronary vessels (Kent *et al.* 1976, Alfonso *et al.* 1974) as well as in skeletal muscle (Kilbom and Wennmalm 1974, Messina *et al.* 1974). The high vascular sensitivity to PGE₂ in the non-visceral tissues observed in the current experiments constitutes a beneficial basis for the significant contribution of endogenous PGs in developing muscle reactive hyperemia.

To summarize, the present investigation demonstrates regional differences in the increase in peripheral resistance in man following administration of the prostaglandin synthetase inhibitor indomethacin. Furthermore, it demonstrates that the degree of vascular relaxation to PGE₂ varies between regions. On the basis of these observations it is suggested that these or organ differences in vascular resistance in man may depend to some extent on locally formed vasodilating PGs.

This study was supported by the Swedish Medical Research Council, project 04X-4341 and by Magnus Bergvalls Stiftelse. Indomethacin was kindly put at our disposal by the Merck Sharp & Dohme Company through its Swedish agent Erik Lindblom & Co KB. PGE₂ was a kind gift from Astra Läkemedel AB.

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Programmed pattern of muscular activity in monkeys landing from a leap

By

ARNE MOSFELDT LAURSEN POUL DYHRÉ POULSEN ALLAN DIERUP and HENRIK JAHNSEN

Muscular activity begins before the foot touches the ground in both stepping (Engberg and Lundberg 1969) and sudden falls (Melvill Jones and Watt 1971 Greenwood and Hopkin 1976, Prochazka *et al* 1977). The activity absorbing the impact of landing, thus, is not elicited by impulses from receptors in the leg. After landing the activity may either be controlled by a program, by local or central reflexes, or it may be of mixed origin. The aim of the study was to determine if reflexes take part in the control of activity occurring immediately after landing.

4 monkeys were trained to jump from one platform to another placed from 50 to 165 cm beneath it. The height of the starting platform in successive leaps was varied in a predetermined random sequence. Thus, a stereotyped movement pattern could not replace regulated onset of anticipatory contraction. Each jump was rewarded with a pellet of food and a recording session consisted of about 40 jumps. The electrical activity of the extensor (*m. triceps brachii*) and flexor (*m. biceps brachii*) was recorded with wire electrodes.

A third recording channel displayed the output from a piezoelectric force transducer in the landing platform. Strain on the wires from the animal to the recording equipment was prevented with contacts rotating in grooves filled with mercury and a spring loaded aluminium rod carried the wires. The difference between the acceleration of the free fall and the acceleration retarded by the wire-carrying device was less than 5%.

Electrical activity in the triceps began 38 ms before landing and the initial extensor burst lasted until about 10 ms after contact with the landing platform. Subsequently 3 or 4 bursts each lasting 12 ms were separated by silent periods of 10 ms. Electrical activity in the biceps began 30 ms before the hands touched the platform but the burst ended 10 ms before contact. Subsequently extensor and flexor activity occurred in a reciprocal pattern (Fig. 1).

The time of onset counted from the time of landing was independent of the height of the jump. The onset thus occurred at greater heights the higher the jump. The probability that the monkey turned the extensor on at a constant time after departure from the starting platform was calculated from linear regression analysis and found to be less than 0.001.

The timing and the pattern of the muscular activity were independent of the height of the jump. This is compatible with the release of a programmed pattern of responses (Chan and Kearney 1977).

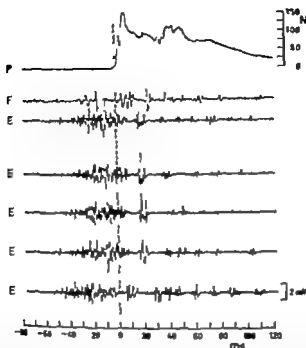


Fig. 1. Leading force (P) and EMG in arm flexor (F) and extensor muscles (E), during leading from a leap. Upper EMG recordings are from leaps from 120 cm. The lowest recording is from a leap to a paper disc of the same height. The monkey went through the paper platform and made a solid landing after jumping 8 cm. Notice that the first burst of extensor activity moved forward in time in relation to the landing, indicating that the monkey was deceived. The first period of silence in the extensor now occurred before landing and thus is out of reflex origin. The second burst of activity also moved forward in time, indicating that it is controlled or influenced by the motor program.

To determine if the first silent period in the extensor discharge was reflex inhibition we used an 8 cm high, solid black cylinder on top of the landing platform and let the monkey jump 10 times. In the subsequent trial the solid cylinder was replaced with another of the same appearance, but made from tissue paper. The lowest recording of Fig. 1 shows that extensor activity now began about 50 ms before the impact on the landing platform. The time, measured from high-speed cinematography for the 8 cm fall from the top of the paper cylinder to the landing platform was 16 ms. This means that the activity started about 35 ms before the hands arrived at the soft paper cylinder. The deception moved not only the time of onset of the extensor forward in time, but the onset of silence moved with it and now occurred before the impact. Hence, this part of the pattern was not a reflex.

The functional significance of the EMG pattern after landing is uncertain. Nichols and his colleagues (1976) found that the reflex mediated peak of EMG activity occurring just after the onset of muscle stretch was associated with a huge increase in stiffness of the lengthening muscle. The basis of comparison of stiffness was the same muscle under steady motorneural excitation. It is possible that the programmed pattern observed by us after landing also is associated with high muscular stiffness.

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Contractile action of a stable prostaglandin endoperoxide analogue on the human umbilical artery

By

TORSTEN TUVENO, KYLL STRANDBERG and MATS HAMBERG

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Prostaglandin (PG) endoperoxides PGG₂ and PGH₂ are potent contractors of the human umbilical artery (Tuveno *et al.* 1976). During PG biosynthesis they are converted to PGs, *e.g.* PGE₂, PGF_{2α} and PGI₂ (prostacyclin) and to thromboxane A₂ (Hamberg *et al.* 1975). As TxA₂ is an even more potent contractor of the human artery than the PG endoperoxides it is possible that the effects produced by the compounds in fact reflect the formation of TxA₂ (Svensson *et al.* 1977). The recent use of 13(S)-hydroxy-9 α ,11 β -(epoxymethano)prosta-5,13-dienoic acid (EPA), a stable endoperoxide analogue, offers a tool for evaluation of the biological effects of the PG oxides *per se*.

In this study we have compared the contractile effects of EPA and PGE₂ on spiral strips of human umbilical arteries by studying the cumulative dose-effect relations for the respective compounds. The strips were prepared immediately after cord clamping, and mounted in baths containing Krebs-bicarbonate-glucose solution at 37°C, pH about 7.4, and pCO₂ approximately 100 mmHg and 40 mmHg respectively (Strandberg and Tuveno 1975). The preparations were allowed to stabilize at the level attained spontaneously before testing was begun. Drugs were added to the bath at 3 min intervals until maximal contraction was produced. Both drugs were dissolved in the salt solution used prior to testing. Administration to the bath EPA produced a prompt, sustained contraction of the human umbilical artery. In contrast the response to PGE₂ was slower in onset. As has been shown (Tuveno *et al.* 1976), the response to the endoperoxides (PGG₂, PGH₂) is also rapid in onset, but less sustained.

Cumulative dose-response curves for EPA and PGE₂ are shown in Fig. 1. ED₅₀ for EPA was approximately $3 \cdot 10^{-6}$ mol/l as compared to about $1.5 \cdot 10^{-5}$ mol/l for PGE₂. The 10-fold difference in activity

of EPA as an extremely potent contractor of the isolated human umbilical artery. In fact it is more potent than all other stable compounds tested, including 5-HT. In view of the present results it is likely that previously recorded responses using the PG endo-

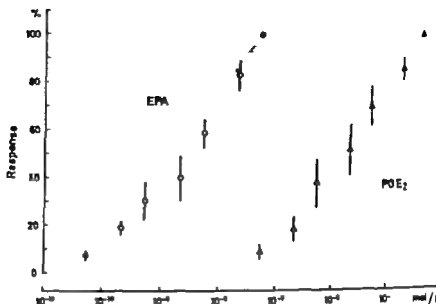


Fig. 1 Cumulative dose response curves for EPA (N=6) and PGE₂ (N=6). Means \pm S.D. are given.

peroxides PGG and PGH at least partly reflect the action of the compounds proper not entirely the conversion product TxA₂.

In analogy with these findings EPA has been reported to be markedly more active than PGG and PGH in a number of other smooth muscle preparations and in raising pulmonary arterial pressure in the cat and the airway insufflation pressure in the guinea (Chapnick *et al* 1976, Hedqvist *et al* 1977).

This study was supported financially by a grant from Stiftelsen Samaritan, Stockholm.

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Abstracts from Meeting of the Scandinavian Physiological Society in Lund 25-26 November 1977

COMMUNICATIONS

C 1

Independent Myogenic Microvascular Control

by P-O GRÄNDE and S. MELLANDER. *Department of Physiology University of Lund, Sweden*

Boyle's hypothesis of enhanced contractile activity in vascular smooth muscle in response to increased blood pressure has received strong support from hemodynamic as well as vital microscopy studies. The described myogenic responses, however, almost exclusively been studied in terms of the active constrictor/dilator responses which develop upon a given steady state increase/decrease in vascular transmural pressure, hence, can be considered to reflect "static" myogenic reactivity.

In addition, a dynamic or rate-sensitive, component in vascular myogenic control has recently been demonstrated *in vitro* by observations of electrical and mechanical activity in a portal vein in response to a given static stretch or shortening applied at various rates (Grände and Mellander 1975). Dynamic stimuli were much more effective in exciting or inhibiting the vascular smooth muscle than static stimuli. This regulatory principle seems to apply to the microvessels *in vivo* as well as revealed by studies on the carotid of skeletal muscle exposed to a given change of transmural pressure applied at different rates (Grände *et al.* 1977).

These stimulus-effector characteristics were analysed in greater detail in the present study by observations of total and microvascular resistance responses to graded changes in vascular transmural pressure (P_T) applied at different rates (dP_T/dt) over the entire range from +7.5 to -7.5 mm Hg/s. The rate-sensitive microvascular responses, developing during the phase of changing P_T , were distinctly graded in relation to the magnitude of the dP_T/dt stimulus, both with regard to the amplitude of the resistance response and the rate of resistance change per unit time (dR_{micro}/dt). The static microvascular responses, noted in the steady state phase of constant increased P_T , were small compared to the dynamic ones and graded in relation to the amplitude of the P_T increase. Rate-sensitivity in microvascular control was bi-directional, causing constriction in response to positive, and dilation in response to negative values of dP_T/dt . The data further indicated that the myogenic receptor units operate with different thresholds. The described rate-sensitivity in the myogenic control system seems to increase its rapidity, stability and sensitivity and thereby can contribute efficiently to well-adapted and refined microvascular control.

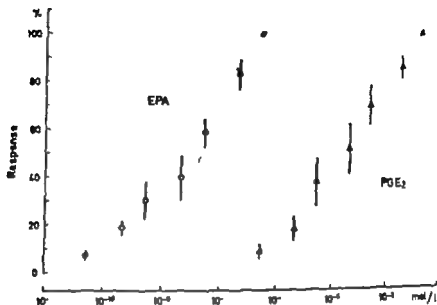


Fig. 1. Cumulative dose response curves for EPA ($N=6$) and PGE₂ ($n=6$). Means \pm S.D. are given.

peroxides PGG and PGH₂ at least partly reflect the action of the compounds proper not entirely the conversion product TxA₂.

In analogy with these findings EPA has been reported to be markedly more active than PGG and PGH₂ in a number of other smooth muscle preparations and in raised pulmonary arterial pressure in the cat and the airway insufflation pressure in the guinea pig (Chapnick *et al* 1976, Hedqvist *et al* 1977).

This study was supported financially by a grant from Stiftelsen Samariten, Stockholm.

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Abstracts from Meeting of the Scandinavian Physiological Society in Lund 25-26 November 1977

COMMUNICATIONS

C 1

Independent Myogenic Microvascular Control

by P.-O. GRÄNDE and S. MELLANDER, Department of Physiology University of Lund, S. edsvä

Louise's hypothesis of enhanced contractile activity in vascular smooth muscle in response to increased blood pressure has received strong support from hemodynamic, organ and vital microscopy studies. The described myogenic responses have almost exclusively been studied in terms of the active constrictor/dilator responses that develop upon a given steady state increase/decrease in vascular transmural pressure, hence can be considered to reflect static myogenic reactivity. An "additional dynamic" or rate-sensitive, component in vascular myogenic control was recently demonstrated *in vitro* by observations of electrical and mechanical activity in a portal vein in response to a given static stretch or shortening applied at various rates (Johansson and Mellander 1975). Dynamic stimuli were much more effective in exerting inhibiting the vascular smooth muscle than static stimuli. This regulatory principle seems to apply to the microvessels *in vivo* as well as revealed by studies on the carotid bed of skeletal muscle exposed to a given change of transmural pressure applied at different rates (Grände *et al.* 1977).

The stimulus-effector characteristics were analysed in greater detail in the present study by observations of total and microvascular resistance responses to graded changes in transmural pressure (P_T) applied at different rates (dP_T/dt) over the entire range from -7.5 to +7.5 mm Hg/s. The rate-sensitive microvascular responses, developing during the phase of changing P_T were distinctly graded in relation to the magnitude of the stimulus, both with regard to the amplitude of the resistance response and the rate of resistance change per unit time (dR_{micro}/dt). The static microvascular responses, developed in the steady state phase of constant increased P_T were small compared to the dynamic ones and graded in relation to the amplitude of the P_T increase. Rate-sensitivity in microvascular control was bi-directional causing constriction in response to positive values of dP_T/dt . The data further indicated that the myogenic receptor units operate with different thresholds. The described rate-sensitivity in the myogenic control system seems to increase its rapidity, stability and thereby can contribute efficiently to well-adapted and refined microvascular control.

Dr. Robert Helig Library

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C 2

Potassium Permeability of the Mesothelium of the Frog Mesentery

By J FRØKJÆR JENSEN and O CHRISTENSEN *Institute of Medical Physiology Dept A The Panum Institute University of Copenhagen Denmark*

The mesothelium covering the mesentery has been claimed to possess diffusional properties equivalent to capillary endothelium and the *in vitro* preparation of the isolated mesentery has been used as a model of the endothelium (Rasio 1974)

We have determined the K^+ permeability of the mesothelium of the frog mesentery *in vivo*. The K^+ -concentration of the interstitial tissue is increased by a continuous perfusion through a single capillary with an isotonic Ringer's solution containing an elevated K^+ concentration. When steady-state is achieved the perfusion is stopped and the wash-out of K^+ across the mesothelium followed by use of K^+ sensitive microelectrodes inserted into the interstitium at various distances from the capillary (Fig. 1)

After an initial lag time the wash-out curves show a monoexponential decline of K^+ concentration. The K^+ -permeability of the mesothelium is then determined from the relation, $P_K = d/\tau$ where τ is the decay time and d is the thickness of the interstitium. We found a $P_K = 2.4 \pm 0.8 \cdot 10^{-8}$ cm sec (S.D. $n=10$) a value about 25 times lower than the K^+ -permeability of the mesenteric capillaries (Crone Frøkjær Jensen Friedman and Christensen 1978) and about 30 times lower than the values previously reported from *in vitro* determinations. This large discrepancy could be due to damage of the mesentery during *in vitro* mounting (Tesi Detraz and Forsmann 1971)

We conclude that in the frog the mesothelium of the mesentery differs significantly from

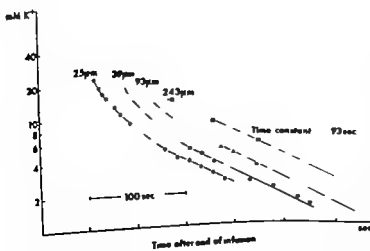


Fig. 1 K^+ wash-out from the mesentery. The decay of the interstitial K^+ concentration is shown at various distances from a perfused capillary through which the interstitium had been loaded with potassium. After an initial lag time the decay in K^+ concentration is monoexponential at all distances. The time constant for wash-out was 93 sec.

critical properties from the endothelium of the capillary and thus represents a poor role of the capillary wall. Furthermore, the findings stress the importance of the mesothelium as a diffusion barrier—a fact which may be critical in experiments on osmotic fluid movement across the capillary wall induced by hypertonic solutions on the surface of the mesothelium (Landis and Sage 1971, Curry, Mason and Michel 1976).

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C 3

Small Capillary Volume in Rat Hindquarter Measured with Gravimetric Method

By A. KAWA, B. REPE and B. POLKOW. Department of Physiology, University of Göteborg, Sweden.

Attempts have been made, particularly with morphological technique, to estimate capillary volume in tissues. In the present study a physiological approach was utilized in an "isogravimetric" rat hindquarter preparation, so arranged that weight changes due to vascular distension or recoil were automatically balanced off. The volume of the "free" exchange vessels could then be deduced from the weight change caused by transient fluid absorption into the capillaries when inflow was suddenly stopped. In matched hindquarter preparations were perfused in parallel with a plasma substitute at constant total flow and maximal dilatation while total weight was continuously recorded. Starting from isogravimetric conditions one of the arterial inflows was suddenly reduced so that the doubled inflow to the other preparation here caused prompt vascular distension followed by steady transcapillary filtration. In the occluded preparation a post-distensional recoil occurred, followed by a transient absorption of tissue fluid into the post-capillary contents. Arterial and venous outflow pressures were initially so set that the transient vascular distension and recoil events largely cancelled out between the two preparations. Upon the unilateral flow interruption the steady filtration in the unoccluded vascular bed showed up first after a distinct delay which was due to the transient fluid absorption in the occluded vascular bed. From this time delay Δt , and the final outflow rate Q , the absorbed fluid amount Q was obtained by $Q\Delta t$. Capillary volume V_c could then be calculated by

$$V_c = Q/\ln[(H_c/H_c - P_c - P_v)]$$

where H_c , P_c , P_v represent plasma colloid osmotic pressure, capillary pressure and venous outflow pressure during control isogravimetric conditions.

Capillary volume thus obtained amounted to 0.55–0.60 per cent of hindquarter weight

where total vascular volume at maximal dilatation is around 3-4 per cent. The approximate capillary number in hindquarter skeletal muscle was also deduced from these data and from average capillary diameter. A figure of 450-500/mm² cross-sectional area was obtained in good agreement with morphological counts in skeletal muscle.

The present approach may be useful particularly in composed tissues of complex design where other methods meet with difficulties and where one wants to know the volume of the "functional" capillary vessels i.e. of the microvascular sections allowing for a substantial exchange across the walls which then includes venular parts.

Supported by the Swedish Medical Research Council Project No B78-14X-0016-14A

C 4

Capillary Permeability: Is the Pappenheimer-Karnovsky Pore Model Correct?

By WILLIAM P. PAASKE *Institute of Medical Physiology B University of Copenhagen Denmark*

Capillary permeability of the hydrophilic indicators ⁵¹Cr-ethylene-diamine-tetraacetate, ⁵¹Cr EDTA, ⁵⁷Co-cyanocobalamin, ⁵⁷Co-B12, and Inulin-(¹⁴C)carboxylic acid was determined in rabbit adipose and cutaneous tissues and cat gastrocnemius muscle as indicated in Table I. The single injection residue detection (alternatively venous sampling) technique and computerized semi on-line black-box analysis were used.

The permeability coefficients P_e were calculated from the capillary diffusion coefficient

TABLE I. Capillary permeability in adipose tissue, cutaneous tissue and skeletal muscle.

		Adipose tissue	Cutaneous tissue	Skeletal muscle
CDC(⁵¹ Cr EDTA)	ml/100 g min	2.0 (n=6)	3.7 (n=6)	3.8-9.1 (n=16)
CDC(⁵⁷ Co-B12)	ml/100 g min	1.1 (n=6)	2.3 (n=6)	1.6-4.4 (n=16)
CDC(¹⁴ C-Inulin)	ml/100 g min			0.84 (n=7)
P_e (⁵¹ Cr EDTA)	cm/s	0.95 $\cdot 10^{-6}$	0.88 $\cdot 10^{-6}$	0.74 $\cdot 10^{-6}$
P_e (⁵⁷ Co-B12)	cm/s	0.52 $\cdot 10^{-6}$	0.56 $\cdot 10^{-6}$	0.45 $\cdot 10^{-6}$
P_e (¹⁴ C-Inulin)	cm/s			0.20 $\cdot 10^{-6}$
CDC(⁵¹ Cr EDTA)/ CDC(⁵⁷ Co-B12)		1.82	1.61	1.79
CDC(⁵¹ Cr EDTA)/ CDC(¹⁴ C-Inulin)				3.21
CDC(⁵⁷ Co-B12)/ CDC(¹⁴ C-Inulin)				2.04
D(⁵¹ Cr EDTA)/ D(⁵⁷ Co-B12)	1.79			
D(⁵¹ Cr EDTA)/ D(¹⁴ C-Inulin)	3.17			
D(⁵⁷ Co-B12)/ D(¹⁴ C-Inulin)	1.76			

Abbreviations: CDC, capillary diffusion coefficient; P_e , permeability coefficient; D, free diffusion coefficient in water at 37°C.

leaks, CDC, and estimates of capillary surface area (adipose tissue: $115 \text{ cm}^2/\text{g}$, cutaneous tissue and skeletal muscle: $70 \text{ cm}^2/\text{g}$)

In skeletal muscle, CDC increased with increasing plasma flow. Considering P constant, a 3-3.5 fold increase ("recruitment") of effective exchange area was found with a plasma flow increase from 4.0 to $52.4 \text{ ml}/100 \text{ g min}$.

The indicators permeated the endothelial barrier of the continuous capillaries at rates proportional to their free diffusion coefficients in water at 37°C . This is taken as evidence for the conclusion that restricted diffusion did not occur.

The findings are in agreement with those obtained in skeletal muscle by the indicator diffusion method (Crone 1963). They imply a much larger pore size than the 30 \AA equivalent pore radius estimate obtained with the osmotic transient method (Pappenheimer et al. 1951) which is complicated by osmotic reflection coefficients corrections. The basic assumption of Pappenheimer of a well-mixed interstitial compartment is not supported by the present series, as the washout of indicator from the interstitium does not follow a monoexponential function.

The Pappenheimer-Karnovsky (1967) concept of aqueous interendothelial parallel plate channels (clefs) with a 37 \AA slit width as the morphological pathway for transcapillary exchange of hydrophilic solutes is not supported by the present findings. The transendothelial channel system formed dynamically by random fusion of intraendothelial vesicles (Salmassi et al. 1975) seems a possible structural equivalent.

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C 5

The Temperature Dependence of the Latency Relaxation in Isolated Single Frog Skeletal Muscle Fibres

By P. HAUGEN Department of biophysics University of Copenhagen, Denmark

The latency relaxation (LR) was studied at sarcomere lengths (S) in the range $2.2-3.7 \mu\text{m}$ and at temperatures from 0 to 23°C in order to shed some light on what processes might be involved in generating this phenomenon.

Materials and methods were basically the same as those previously described (Haugen and Sørensen, 1976). The LR was quantified in terms of the values defined in the text of Fig. 1.

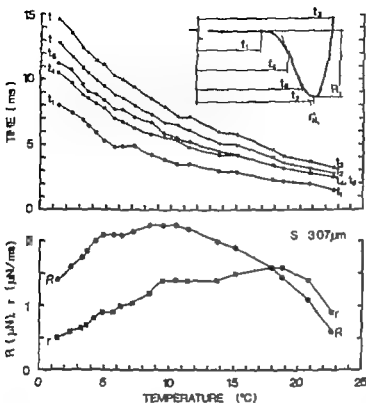


Fig. 1 Explained in the text.

Two types of experiments were performed. 1) the values were measured as functions of S while the fibre was kept at constant temperature. R passed through a maximum which occurred at higher S the lower the temperature. r_s showed a similar dependence on S but its maximum was less sharp than that of R . At temperatures below about 12° the portion (from t_4 to t_5) of the falling phase was linear. At higher temperatures t_4 and t_5 merged into an inflexion point. This can be interpreted as if LR originates from a lengthening process which eventually is masked by the contraction. At all temperatures intervals from t_1 to t_2 , t_2 to t_3 , and t_3 to t_4 were proportional to the distance from the Z-line to overlap zone thereby indicating that the lengthening originates from the thin filament t_4 when separate from t_5 was independent of S up to about $3 \mu\text{m}$ while it increased with S above that. 2) The values were measured as functions of temperature at constant S . The results of such an experiment are shown in Fig. 1. The processes had a Q_{10} of about 2 as obtained from the time parameters. R and r_s have maxima at 9 and 19°C respectively.

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C 6

Sarcomere Behaviour during Isometric Relaxation in Isolated Striated Muscle Fibres

By K. A. P. EDMAN and F. W. FLITNEY *Department of Pharmacology University of Lund, Sweden*

cardiac muscle fibres of *R. temporaria* were stimulated to produce fused isometric twitches (plate electrodes, 0.2 ms square pulses 0–20°C) and the changes in sarcomere length were followed by streak photography of the diffraction spectra produced by illuminating the fibre with a laser (He Ne 1 mm beam width). Streak recordings were made at successive 1 mm intervals along the entire length of the fibres.

The sarcomeres in some segments were seen to shorten during relaxation, at the expense of those in other regions which elongated (Edman and Flitney 1977 and for further references). In about 30% of the segments studied the first order line fragmented into several discrete maxima, and the individual microstriations were often observed in cross section revealing a tendency for sarcomeres having lengths marginally greater than the mean to shorten, and vice versa. The pattern of sarcomere movements remained constant in any one fibre. Regions which elongated were found at all possible locations but occurred more frequently near the fibre ends. The onset of the changes in length coincided with the 'shoulder' on the tension record, and the maximum displacement occurred at a time when tension approached zero (about 2% of maximum tetanic force at 2.20 μ m sarcomere length).

The peak amplitude of the sarcomere elongation (ΔS_e) exceeded the amplitude of sarcomere shortening (ΔS_s) by an amount which depended upon the fraction of the fibre which shortened (0.71 ± 0.10 mean \pm S.D.) and the accompanying change in length of the series elastic element ($2.01 \pm 1.38\%$ fibre length). Both ΔS_e and ΔS_s and the mean velocity of the length change, decreased with increasing sarcomere length (range: 1.9–3.2 μ m).

The pattern of length changes suggest that the duration of mechanical activity differs along the length of the fibre, being shortest in those segments which elongate during relaxation. A possible explanation for this is that the amount of calcium released, and/or the rate at which it is re-sequestered varies in different fibre segments. As a result some segments will remain active over a longer time and undergo further shortening during the tension decay stretching weaker regions which ultimately yield (at the 'shoulder' of the tension record) when the force per cross-bridge exceeds a critical value.

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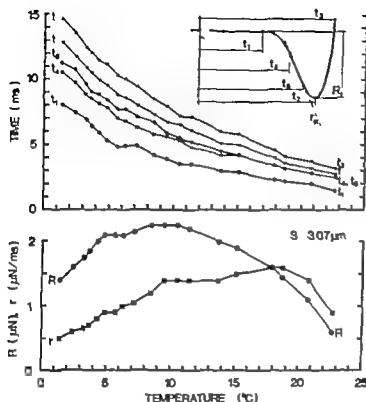


Fig. 1 Explained in the text.

Two types of experiments were performed. 1) the values were measured as functions of S while the fibre was kept at constant temperature. R passed through a maximum which occurred at higher S the lower the temperature. r_m showed a similar dependence on S but its maximum was less sharp than that of R . At temperatures below about 12° the portion (from t_1 to t_2) of the falling phase was linear. At higher temperatures t_1 and t_2 merged into an inflexion point. This can be interpreted as if LR originates from a lengthening process which eventually is masked by the contraction. At all temperatures intervals from t_1 to t_2 , t_2 and t_3 were proportional to the distance from the Z-line to overlap zone thereby indicating that the lengthening originates from the thin filament t_1 , when separate from t_2 was independent of S up to about $3 \mu\text{m}$ while t_2 increased with S above that. 2) The values were measured as functions of temperature at constant S . The results of such an experiment are shown in Fig. 1. The processes had a Q_{10} of about 2 as obtained from the time parameters. R and r_m have maxima at 9° and 19°C respectively.

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C 8

The Timing of Muscular Contraction in Monkeys Landing from a Leap

By A. MØRFELDT LAURSEN, P. DYHRE POULSEN, H. JAHNSEN and A. DJØRUP *Institute of Neurophysiology, University of Copenhagen, Denmark*

Muscular activity begins before the foot touches the ground in both stepping and sudden falls. The activity absorbing the impact of landing, thus, is not elicited by impulses from receptors in the leg. To investigate the mechanism controlling the onset of anticipatory muscular activity we have studied the electromyogram of extensors and flexors in monkeys jumping from different heights.

Forces on the landing platform and acceleration of the head were both maximal 10–15 msec after the fingers touched the platform. The acceleration of the head associated with landing was brief, confined to about 10 msec and it was a linear function of the height of the jump (3 g/m). At the moment of peak acceleration the arms were in nearly full extension and during the subsequent flexion of the arms acceleration of the head was absent. A second peak, one fourth the size of the first, occurred after about 55 msec when the head rose toward the position associated with normal posture.

Electrical activity in the triceps began 44 msec before landing. The initial extensor burst lasted until about 19 msec after contact with the landing platform and subsequently 3 or 4 bursts, each lasting 12 msec were separated by silent periods after about 10 msec. Electrical activity in the biceps began 32 msec before the hands touched the platform but the burst ended 8 msec before contact. Subsequently extensor and flexor activity occurred in a reciprocal pattern.

The time of onset and the pattern of muscular activity were independent of the height of the jump. The onset thus occurred at greater heights the higher the jump. The probability that the monkey turned the extensor on a constant time after departure from the starting platform, as calculated from linear regression analysis and found to be less than 0.001.

We suggest that most or all of the motor units in the extensor fire in synchrony to stiffen the arm, as they did during a forceful ballistic contraction studied by Desmedt and Godaux (1971) and that the activity in the flexor ceases by reciprocal inhibition when most or all of the motor units in the extensor fire in synchrony.

C 9

Receptor Mechanisms in the Control of Smooth Muscle Activity in the Human Oviduct

By B. LINDBLOM, L. HANBERGER and B. LJUNG *Department of Physiology and Obstetrics & Gynecology, University of Göteborg, Sweden*

Transport of the ovum through the human oviduct is assumed to be influenced physiologically by both hormonal and neuronal factors. A muscular "tube-locking" mechanism under tonic control has been shown to exist in the ampullary-isthmic junction of the rabbit oviduct (Brunden 1964; Howe and Black 1973) and certain observations (Sjöberg 1967

Phase Dependent Modulation of the Transmission in Reflex Pathways during Fictive Locomotion

By O. ANDERSSON, H. FORSSBERG and M. LINDQUIST, *Department of Physiology III Karolinska Institutet, Stockholm, Sweden*

In walking spinalized cats a tactile stimulation of the dorsum of the paw evokes a flexor reaction during the swing phase but an increased extensor activity during the support phase (Forssberg *et al.* 1975, 1977). The aim of the present investigation has been to analyze the neural mechanisms of this "phase dependent reflex reversal". To summarize three different models could contribute to the reversal: 1. The cyclical changes in the excitability level of the motoneurone that occur concomitant with the "locomotor commands". 2. A phasic depression or facilitation of the transmission in the reflex pathways to flexors and extensors a) by interaction from other peripheral pathways or b) by influence from the spinal "locomotor generator" responsible for the basic locomotor movements (see Forssberg *et al.* 1977).

A preparation with "fictive locomotion" was used. Adult cats were acutely spinalized, paralyzed (tubocurarin) and injected i.v. by DOPA and Nialamid drugs which activate the "locomotor generator" (Grillner and Zanger 1974). The alternating locomotor activity was recorded from extensor and flexor muscle nerve filaments.

A weak electrical stimulation of the paw (2 mA, 0.5 ms) evoked large responses in the nerve filaments. During flexion there was large flexor responses and during extension large extensor responses. Hence the "reversal" present in "walking" immobilized animals eliminated the possibility of phasic peripheral interaction (2a) as exclusive origin for the modulation.

By intracellular recordings from motoneurons it was possible to distinguish between the two remaining models. In flexor motoneurons the paw stimulation evoked an EPSP after about 10 ms in some neurons followed by an IPSP. In 16 of 20 motoneurons the EPSP was larger during flexion than during extension and in 8 of these significantly larger ($p < 0.05$, T-test). Generally in the extensor motoneurons the size of the responses was smaller and the modulating effect less pronounced than in the flexor motoneurons.

The results show that there is a phasic modulation of the transmission in different reflex pathways during "fictive locomotion". The effectiveness of different reflex pathways is thus controlled by the "spinal locomotor generator"—a phasic central gain control has been demonstrated.

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the amine level remains constant until the last two weeks during which it falls to the low values as in the contralateral fetus-containing horn (Owman *et al.* 1975).

In the present investigation the mechanical responses to electrical field stimulation studied in isolated myometrial strips from virgin guinea pigs pre-treated with estradiol and estradiol and 6-OH-dopamine and from pregnant guinea pigs. The uterine strips contracted either spontaneously or by adding methacholine or bradykinine to the bath. The adrenergic innervation of the uterus was also studied histochemically and the noradrenaline content measured fluorometrically.

Electrical stimulation caused a relaxation of the uterus from non-pregnant guinea pigs along the tone about 30%. Using hexamethonium, guanethidine, tetrodotoxin and reserpinol it was shown that the responses were due to activation of postganglionic sympathetic nerves acting on β -adrenoceptors. Myometrial strips taken from pregnant guinea pigs about two weeks before term and strips from animals pretreated with 6-OH-dopamine showed no response to the stimulation. Addition of noradrenaline caused relaxation of both the pregnant and the non-pregnant uterus mounted in the same organ bath. Threshold doses were lower and the responses greater in the pregnant uterus possibly indicating a supersensitivity to noradrenaline. When using animals with unilateral pregnancy the strips from the empty uterine horn were relaxed by electrical stimulation, while strips from the contralateral fetus-containing horn mounted in the same bath showed no response.

In the non-pregnant guinea pigs there was a rich occurrence of adrenergic nerves in the myometrium, while no adrenergic uterine nerves could be seen in the pregnant animals or in animals treated with 6-OH-dopamine. The tissue content of noradrenaline was reduced by the treatment with 6-OH-dopamine to about 5% of controls.

It is concluded that the morphological disappearance of adrenergic nerve fibres and the reduction of transmitter content in the guinea pig uterus during pregnancy is accompanied by a loss of adrenergic nerve function possibly resulting in a denervation supersensitivity to the transmitter.

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C 11

Muscle Metabolites during Recovery after Maximal Exercise in Man

By O. VAAGLE, E. NERHOLME, O. GRONNEROD and L. HERMANSEN. *Institute for Medical Physiology, Oslo, Norway.*

In a recent study it was shown that only a small fraction of the lactate produced in skeletal muscle during maximal exercise disappeared from muscle into circulation during recovery. In our observation and our findings of a rapid lactate disappearance in muscle, a rapid

Owman *et al* 1967 1976) give evidence for a similar arrangement in the human oviductal isthmus. The timing of ovum transfer from the ampulla of the oviduct to the uterus might thus be regulated by the degree of activation of excitatory and inhibitory adrenoceptor mediated mechanisms in the smooth muscle of the tubal wall. To classify these receptors in the α - and β -types, small strips from the circular (inner) and longitudinal (outer) smooth muscle layers in the isthmus part of human Fallopian tubes were used for studies of isometric contractile activity in organ baths.

Addition of adrenaline (A) or noradrenaline (NA) at a concentration of 1–10 μ M resulted in a marked increase in the spontaneous phasic activity of the longitudinal specimens, while circular preparations consistently were inhibited. The inhibitory response to A and NA was reversed into a slight increase in activity after β -blockade with propranolol (30 μ M). The same type of response was elicited after addition of an α -adrenergic agonist phenylephrine (1–30 μ M). Isoprenaline (1–100 nM) markedly inhibited the contractility in both circular and longitudinal preparations.

The β_2 -agonist terbutaline caused no alteration of contractility in concentrations lower than 0.3 μ M. With higher concentrations of terbutaline (0.3–10 μ M) a gradually diminished activity was obtained in both circular and longitudinal specimens.

It is concluded that the adrenoceptors in the circular muscle layer of the human oviduct are predominantly inhibitory of the β -type. In the longitudinal muscle layer the receptors are mainly of the α -type. This differentiation in distribution of α - and β -receptors in the two muscle layers may play a functional role in the physiological control of ovum transport.

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C 10

Disappearance of Adrenergic Innervation in the Guinea Pig Uterus during Pregnancy

By M. ELMÉR, P. ALM and G. THORBERT. *Institute of Physiology, Histology and Obstetrics and Gynecology, University of Lund, Sweden*

The smooth musculature in the uterus of the guinea pig is innervated by adrenergic nerves and the content of neuronal noradrenaline, measured fluorometrically, varies during pregnancy and under the influence of sex hormones (Sjöberg 1968). In animals with unilateral pregnancy the content of noradrenaline in the pregnant uterine horn is continuously reduced, reaching near zero levels at term. In the empty

C 12

Medullary Control of Muscular Glycogenolysis and Pancreatic Hormonal Secretion during Exercise in Rats

By E. A. RICHTER, B. SONNE, J. J. HOLST and H. GALBO. *Institute of Medical Physiology B and Institute of Medical Physiology C University of Copenhagen Denmark*

It has previously been reported (Galbo *et al.* 1977) that in adrenodemedullated rats which have been injected with 6-hydroxydopamine—a drug that selectively destroys sympathetic terminals (Tjornehoj and Tranzer 1968)—muscular glycogen depletion as well as glucagon secretion are decreased during prolonged exercise whereas insulin secretion is intact compared to the findings in control rats. In the present study we have elucidated the effects of sympathectomy could be ascribed selectively to the lack of either the adrenal medulla or of the peripheral sympathetic nerve endings.

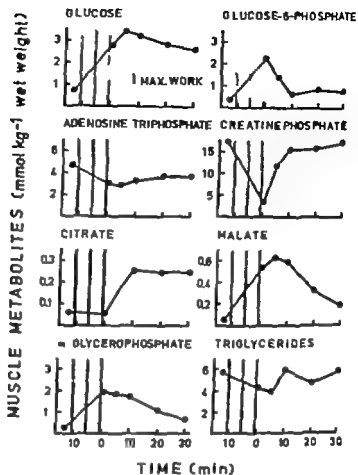
Groups of eight rats were either adrenal demedullated (DM) injected with 6-hydroxydopamine (6-OHD) adrenal demedullated and injected with 6-hydroxydopamine (SX) or sham operated and sham injected controls (C). The rats either rested or were forced to swim with a tail weight (2% of body weight) for 100 min in tepid water. Subsequently they were decapitated by cardiac puncture and samples from the soleus and the deep part of the vastus lateralis muscle were collected.

During exercise the plasma concentrations of insulin were higher and plasma glucagon concentrations were lower in SX and DM-rats compared to C and 6-OHD-rats. Blood glucose concentrations increased only in 6-OHD (25%) and C-rats (61%). During exercise muscle glycogen concentrations in the soleus muscle decreased 50% and 54% in 6-OHD- and C-rats respectively ($P < 0.05$). In the deep part of the vastus lateralis the decreases in glycogen concentrations were 44% and 41% respectively ($P < 0.05$). However in DM and SX-rats the markedly reduced decreases were: 9% and 0% respectively ($P > 0.05$) in the soleus muscle and 26% ($P < 0.05$) and 9% ($P > 0.05$) respectively in the deep part of the vastus lateralis muscle.

It is concluded that in exercising rats the adrenal medullary hormones significantly affect muscular glycogen depletion as well as pancreatic α -cell secretion and significantly inhibit β -cell secretion. The peripheral sympathetic nerves however only play a minor role in the control of muscular glycogen depletion and pancreatic hormonal secretion during exercise.

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muscle glycogen resynthesis and almost no uptake of glucose by the muscles during recovery were offered in support of Meyerhof's original hypothesis of a conversion of lactate into glycogen in skeletal muscle. To further test this hypothesis, changes in the concentrations of adenosine triphosphate (ATP), creatine phosphate (CP), triglycerides and different glycolytic intermediates were measured in the quadriceps muscle of 15 healthy male subjects at rest before intermittent maximal exercise and at different time intervals during the recovery period (30 min). The results (Fig. 1) indicated that changes in the glycolytic intermediates could account for only a small fraction of the glycogen synthesized during the recovery period. The glucose and glucose-6-phosphate concentrations during recovery are consistent with an inhibition of hexokinase and the small uptake of glucose by muscle reported earlier. Moreover, the concentrations of ATP, CP and citrate observed during recovery suggest that phosphofructokinase is also inhibited. Altogether, the results of the present investigation are consistent with the hypothesis of a conversion of lactate into glycogen in human skeletal muscle.

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C 14

Effect of Chronotropic and Inotropic Effects on Stroke Volume Regulation in the Dog

LIJBECK, J. LEKVEN and F. KIL. *University of Oslo Institute for Experimental Medical Research Ullevaal Hospital Oslo Norway*

As a rise in heart rate is combined with increased sympathetic activity. The present study was undertaken to separate influences of changes in chronotropy and inotropy on stroke volume.

Heart rate was increased in steps by right atrial pacing in open-chest dogs at control level during raised inotropy by continuous isoproterenol infusion (0.20-0.25 $\mu\text{g/kg}$) and during reduced inotropy by propranolol administration (0.5 mg/kg). Left ventricular dimensions were studied by continuously recording myocardial chord lengths (MCL) in ultrasonic elements sewn into the anterior wall of the left ventricle (Bjuggeldahl *et al.* 1969).

At any level of inotropy stroke volume declined linearly to increments in heart rate. At constant heart rate a rise in inotropy increased stroke volume and stroke volume declined after propranolol administration. The difference in stroke volume at the various levels of inotropy was not influenced by heart rate.

End-diastolic MCL, an estimate of end-diastolic left ventricular volume, was a function of heart rate and not of inotropy. End-diastolic MCL declined linearly to increments in heart rate with the same slope at any level of inotropy. Changes in inotropy at constant heart rate did not influence end-diastolic MCL, except at very high heart rates. End-diastolic MCL was slightly higher during isoproterenol infusion than at control inotropy.

End-systolic MCL, an estimate of end-systolic left ventricular volume, varied greatly with changes in inotropy so that a difference in end-systolic volume could account for the changes in stroke volume. End-systolic MCL decreased only slightly as heart rate was increased almost equally at any level of inotropy. Isoproterenol reduced end-systolic MCL and increased stroke volume whereas propranolol had the opposite effect at constant heart rate.

In conclusion, chronotropic changes mainly influence end-diastolic left ventricular dimensions, whereas inotropic changes mainly influence end-systolic dimensions. Chronotropy and inotropy therefore affect stroke volume regulation through virtually independent mechanisms.

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Left Ventricular Hypertrophy in Spontaneously Hypertensive Rats (SHR)—Consequences of and Compensation for the Altered Frank-Starling Relationship

By E NORESSON ■ E. RICKSTEN M. HALLBÄCK and P. THOREN *Department of Physiology University of Göteborg Sweden*

The degree of left ventricular hypertrophy in hypertension is mainly a consequence of raised afterload but the functional importance has been subjected to considerable debate. The common decrease of stroke volume with progressing hypertension and cardiac hypertrophy may suggest impaired myocardial performance. Further, in isolated perfused SHR hearts the Frank-Starling curve is displaced to the right of that of controls (NCR). However, this displacement appears to be a consequence of reduced diastolic compliance, leading to a lower degree of myocardial prestretch in SHR for a given filling pressure (Hallbäck *et al.* 1975). The maintenance of equal stroke volumes in SHR and NCR *in vivo* would then call for either increased inotropism or/and raised filling pressure in SHR.

To further explore this situation left atrial catheters were chronically implanted in 7 pairs of matched NCR and SHR. Left atrial pressure was measured in the end-expiratory phase being largely twice as high in SHR as in NCR (10.3 ± 0.4 mm Hg ± 0.3 mm Hg and 4.6 ± 0.3 mm Hg respectively, $p < 0.001$).

For additional analysis of the hemodynamics during left ventricular hypertrophy the Frank-Starling relationship was studied at various levels of constant afterload in 17 pairs of SHR and NCR hearts. The paced hearts were perfused *in vitro* in an antegrade perfusion system. Preload and afterload could be independently varied during recordings of cardiac output. At equal afterloads below 100 mm Hg the SHR Frank-Starling curve was displaced to the right of the NCR one at "physiological" preloads and maximal stroke volumes were largely the same. With higher afterloads the SHR hearts maintained their maximal stroke volume considerably better than the NCR ones. However, the SHR curves were still displaced to the right of the NCR ones in the lower preload range though crossing over at higher filling pressures.

Thus, the hypertrophic adaptation of the SHR heart has at least two important hemodynamic consequences. At high afterloads it is certainly able to better maintain stroke volume than the NCR heart, provided the end-diastolic pressure is high enough for optimal prestretch. Nevertheless, due to the reduced diastolic compliance of the thicker wall, the SHR left ventricle *in vivo* can in the ordinary range of filling pressure maintain the same stroke volume as NCR only if the filling pressure is kept higher or/and inotropism is enhanced.

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C 14

Effects of Chronotropic and Inotropic Effects on Stroke Volume Regulation in the Dog
 A. LERBEK, J. LERVEN and F. KIL, *University of Oslo Institute for Experimental Medical Research Ullevål Hospital Oslo Norway*

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Heart rate was increased in steps by right atrial pacing in open-chest dogs at control level during raised inotropy by continuous isoproterenol infusion (0.20-0.25 µg/kg) and during reduced inotropy by propranolol administration (0.5 mg/kg). Left ventricular dimensions were studied by continuously recording myocardial chord lengths (MCL) from ultrasonic elements sewn into the anterior wall of the left ventricle (Bugge-riksen *et al.* 1969).

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C 15

Mechanisms Involved in Transcapillary Fluid Movement into the Secreting Cat Submandibular Gland

By J LUNDVALL and J HOLMBERG *Department of Physiology and Biophysics University of Lund Sweden*

An attempt was made to elucidate the mechanisms involved in the process of net transcapillary fluid movement from the intravascular to the extravascular space of the secreting submandibular gland of the cat. The capillary hydrodynamic conductivity (CFC) in the resting gland was found to be $0.30-0.35 \text{ ml/min} \times 100 \text{ g tissue} \times \text{mm Hg}$ and it increased upon parasympathetic stimulation (20 Hz) in rough relation to the nerve excitation rate to a maximal value of about $1.0 \text{ ml/min} \times 100 \text{ g} \times \text{mm Hg}$. Comparison of the CFC in states of maximal vasodilatation evoked by chorda stimulation and by papaverine infusion indicated that the increase of CFC during parasympathetic activation entirely could be ascribed to an increase of the functional capillary surface area available for transcapillary fluid exchange whereas the specific capillary permeability remained constant. Capillary hydrostatic pressure (P_c) in the resting gland was about 20 mm Hg and increased by 18 mm Hg on the average during maximal vasodilatation produced by chorda activation. Roughly 65% of the total transcapillary fluid transfer from blood into the secreting gland could be ascribed to filtration caused by the increase of P_c . It is suggested that the remaining 35% of the fluid transfer was caused by osmosis resulting from regional tissue hyperosmolality in the active gland. The tissue hyperosmolality as traced in the venous effluent, averaged $18 \text{ mOsm/kg H}_2\text{O}$ at 20 Hz and was found mainly due to increased concentrations of sodium and lactate.

C 16

Secretory Potentials Evoked by Sympathetic Stimulation of the Parotid Gland of Cat

By P THESLEFF N EMMELIN and W GRAMPP *Department of Physiology and Biophysics University of Lund Sweden*

Ekström and Emmelin (1974) have demonstrated that sympathetic stimulation of the parotid gland of cat gives rise to a β -mediated salivary secretion. The corresponding electrophysiological events in the secretory cells were however largely unknown. To investigate these cats were anaesthetized with chloralose, their parotid glands exposed and cells down to a depth of 1.0-1.5 mm impaled by KCl or K citrate-filled bevelled microelectrodes with resistances ranging between 10 and 15 megohms. Recorded signals were processed using conventional electrophysiological techniques. For stimulation of the parotid gland supramaximal shocks as judged by pupillary dilatation or salivary secretion were applied to the sympathetic trunk or auriculo-temporal nerve.

In the great majority of cells impaled by either KCl or K citrate-filled electrodes stimulation of the sympathetic trunk gave rise to a membrane depolarisation which was ac

expressed by a marked decrease in membrane resistance. Both the latency rise time and plateau of this depolarisation were dependent on stimulation frequency. It thus became able at minimal frequencies of 0.1–0.2 Hz and then grew with increasing frequency reaching, at about 5 Hz, a maximal size of about 20 mV in cells with a normal resting potential 35–40 mV. At the same time the latency and rise time of the depolarisation were short of from several tens of seconds to 1–3 s and 5–8 s, respectively. These times could be rather reduced by additional increases in stimulation frequency. At moderate stimulation frequencies the depolarisation was maintained throughout stimulation and at supramaximal stimulation frequencies it outlasted stimulation by several seconds before declining gradually.

Both depolarisation and decrease in membrane resistance due to sympathetic stimulation were abolished or greatly reduced by i.v. injection of practolol (2 mg/kg). Neither of these effects was however influenced by dihydroergotamine (0.5–1.0 mg/kg) or by ropivacaine (200 µg/kg) which was able to completely abolish short latency hyperpolarising secretory potentials evoked by auriculo-temporal nerve stimulation.

As an additional observation it was noted that in part of the cells investigated the depolarisation due to sympathetic stimulation was preceded by a short latency hyperpolarising transient which was abolishable by atropine.

In conclusion, sympathetic stimulation gives rise to a β -mediated depolarisation which presumably results from altered ionic permeabilities of the membrane. Also there seems to be some interaction between sympathetic and parasympathetic innervation which may be of importance in the control of the gland's secretory activity.

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C 17

Inhibition of Gastric HCO_3^- Secretion by Noradrenaline and Adrenaline

By GUNNAR FLEMYSTROM, Department of Physiology and Medical Biophysics, University of Uppsala, Sweden

Active secretion of HCO_3^- by the surface epithelial cells was recently demonstrated in gastric antral and fundic mucosa *in vitro*. The rate of this secretion is approximately 10 percent of maximal H^+ secretion in the same species. It is stimulated by carbamylcholine, db-cGMP and Ca^{++} but not affected by histamine, gastrin or db-cAMP and shows higher sensitivity than H^+ secretion to acetazolamide. A very similar gastric secretion of HCO_3^- has been demonstrated also in the guinea pig *in vivo*.

The function of gastric HCO_3^- secretion may be to reduce H^+ concentration in the immediate vicinity of the luminal surface of the gastric epithelium and thus act as a protective mechanism against acid. Acetazolamide which inhibits HCO_3^- secretion also decreases

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By J LUNDVALL and J HOLMBERG *Department of Physiology and Biophysics
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By P THESLEFF, N EMMELIN and W GRAMPP *Department of Physiology and Biophysics
University of Lund Sweden*

Ekström and Emmelin (1974) have demonstrated that sympathetic stimulation of the parotid gland of cat gives rise to a β -mediated salivary secretion. The corresponding electrophysiological events in the secretory cells were however largely unknown. To investigate these cats were anaesthetized with chloralose, their parotid glands exposed and cells down to a depth of 1.0-1.5 mm impaled by KCl or K citrate-filled bevelled microelectrodes with resistances ranging between 10 and 15 megohms. Recorded signals were processed using conventional electrophysiological techniques. For stimulation of the parotid gland supramaximal shocks as judged by pupillary dilatation or salivary secretion were applied to the sympathetic trunk or auriculo-temporal nerve.

In the great majority of cells impaled by either KCl or K citrate-filled electrodes stimulation of the sympathetic trunk gave rise to a membrane depolarisation which was ac

compensated by a marked decrease in membrane resistance. Both the latency rise time and amplitude of this depolarisation were dependent on stimulation frequency. It thus became variable at luminal frequencies of 0.1–0.2 Hz and then grew with increasing frequency reaching, at about 5 Hz, a maximal size of about 20 mV in cells with a normal resting potential of 15–40 mV. At the same time the latency and rise time of the depolarisation were shortened from several tens of seconds in 1–3 s and 5–8 s respectively. These times could be further reduced by additional increases in stimulation frequency. At moderate stimulation frequencies the depolarisation was maintained throughout stimulation and at supramaximal stimulation frequencies it outlasted stimulation by several seconds before declining gradually.

Both depolarisation and decrease in membrane resistance due to sympathetic stimulation were abolished or greatly reduced by i.v. injection of practolol (2 mg/kg). Neither of these effects was however influenced by dihydroergotamine (0.5–1.0 mg/kg) or by atropine (200 µg/kg) which was able to completely abolish short latency hyperpolarising secretory potentials evoked by auriculo-temporal nerve stimulation.

As an additional observation it was noted that in part of the cells investigated the depolarisation due to sympathetic stimulation was preceded by a short latency hyperpolarising transient which was abolishable by atropine.

In conclusion, sympathetic stimulation gives rise to a β -mediated depolarisation which presumably results from altered ionic permeabilities of the membrane. Also there seems to be some interaction between sympathetic and parasympathetic innervation which may be of importance in the control of the gland's secretory activity.

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C 17

Inhibition of Gastric HCO_3^- Secretion by Noradrenaline and Adrenaline

By GUNNAR FLEWSTROM, Department of Physiology and Medical Biophysics, University of Uppsala, Sweden

Gastric secretion of HCO_3^- by the surface epithelial cells was recently demonstrated in gastric antral and fundic mucosa *in vitro*. The rate of this secretion is approximately 10 percent of maximal H^+ secretion in the same species. It is stimulated by carbamylcholine, b-GMP and Ca^{++} but not affected by histamine, gastrin or db-cAMP and shows higher sensitivity than H^+ secretion to acetazolamide. A very similar gastric secretion of HCO_3^- has been demonstrated also in the guinea pig *in vivo*.

The function of gastric HCO_3^- secretion may be to reduce H^+ concentration in the immediate vicinity of the luminal surface of the gastric epithelium and thus act as a protective mechanism against acid. Acetazolamide which inhibits HCO_3^- secretion also decreases

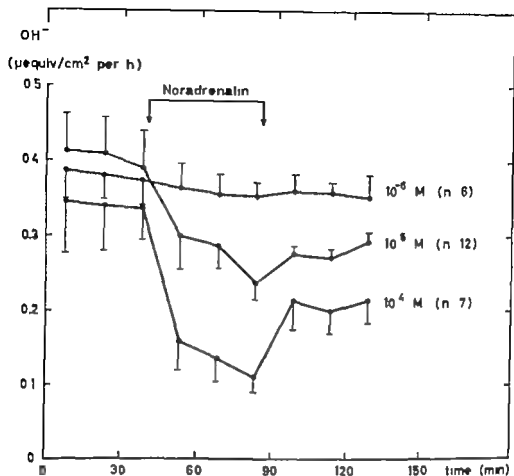


Fig. 1 Effects of some concentrations of noradrenaline on luminal alkalinization in the *Rana temporaria* fundus. Means \pm S.E. are given. None of the concentrations caused significant changes in transepithelial electrical resistance or potential difference. The histamine H₂ receptor antagonist Metiamide (10^{-8} M) was used throughout the experiments to inhibit H₂ secretion.

the ability of the gastric epithelium to resist luminal acid. This made it of interest to study the effects of some catecholamines on gastric HCO_3^- secretion.

Frog (*Rana temporaria*) or *Necturus fundic* or antral mucosae were mounted in *in vitro* chambers. Antrum secreted HCO_3^- spontaneously while histamine H₂ receptor antagonists were used in fundus to inhibit H₂ secretion and thus reveal alkalinization.

Noradrenaline (10^{-8} M) inhibited HCO_3^- secretion both in fundus (Fig. 1) and in antrum. Adrenaline had a similar effect but a higher concentration (10^{-4} M) was required while isoprenaline (10^{-3} M) was without effect. Pretreatment with phentolamine prevented the effect of noradrenaline. At the concentrations used here the catecholamines did not affect gastric H^+ secretion.

The results suggest that the inhibitory effect of catecholamines on HCO_3^- secretion is mediated via α receptors. Sympathetic activation may decrease the ability of the gastric epithelium to resist acid but leave the production of acid intact.

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C 18

Influence of Salivary Secretion, Passive and Active Potassium Transport on Flow Rate in Perfused Submandibular Gland

By L. P. LAUGESEN, J. O. D. NIELSEN and J. HEDEMARK POULSEN *Institute of Medical Physiology Dept A University of Copenhagen The Panum Institute Blegdamsvej 3 C DK-2200 Copenhagen N Denmark*

Perfused salivary glands have been used as models to study electrolyte and water transport in exocrine glands. However, it appears that while the secretory rate in response to muscarinic stimulation is very similar during autoperfusion as compared to perfusion with Locke solution (Poulsen 1975) the rates of the stimulation-induced passive loss of intracellular potassium in the perfusate as well as the post-stimulatory active reuptake of potassium are substantially higher during artificial perfusion than during autoperfusion (Lange 1956; Langeren, Nielsen & Poulsen 1976). To establish whether these differences could be due to the difference between the high flow of saline in the artificially perfused gland and the much lower flow of plasma water in the autoperfused glands, we have studied the effect of the flow rate on secretory rate, passive potassium loss and active potassium reuptake.

Cat submandibular glands were perfused with phosphate-buffered, oxygen saturated Locke solution at 37°C. The glands were stimulated with acetylcholine (10^{-4} M) for 90 s. The flow of perfusion fluid was varied by changing the arterial hydrostatic pressure between 90 and 50 mmHg. The potassium concentration of the venous effluent was measured by flame photometry and the secretory rate was determined gravimetrically.

The secretory rate was found to be largely independent on the flow of venous perfusion fluid in the range 5-17 ml/min. By contrast the rate of the passive stimulation-induced loss of intracellular potassium increased proportionally to the flow in the range 3-8 ml/min, while a further increase in flow only caused a very moderate increase in the rate of active potassium loss. Surprisingly the rate of the active post-stimulatory reuptake of potassium from the perfusion fluid increased proportionally to the flow within the whole range studied (3-18 ml/min).

It is concluded that the very high rates of passive and active potassium transport between salivary gland cells and perfusion fluid observed during artificial perfusion can be ascribed to the supraphysiological high vascular flow rate of water and electrolytes. The

experiments also demonstrate that the active transport mechanism responsible for the post stimulatory reuptake of potassium is very far from being saturated under physiological conditions

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C 19

Functional Interaction between Fish Hemoglobin Erythrocytic Nucleoside Triphosphates and Magnesium

By ROY E. WEBER *Department of Zoophysiology University of Aarhus*

Recent studies (Wood & Johansen 1972 Geoghegan & Poluhowich 1974 Weber *et al* 1975) have shown that the nucleoside triphosphates (NTP) ATP and GTP (guanosine triphosphate) are allosteric effectors that continuously adapt the O_2 affinity of hemoglobin

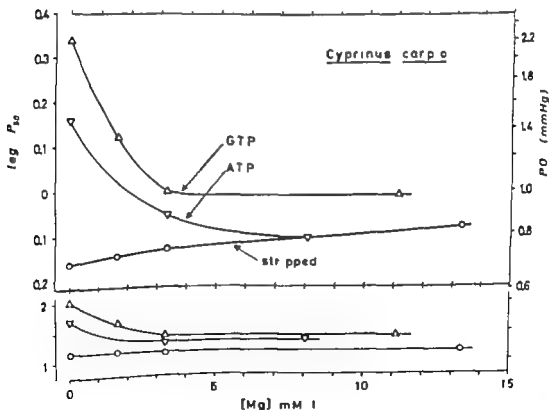


Fig. 1 The effect of magnesium ion on the half-saturation O_2 tension P_{50} , and Hill's coefficient n of "stripped" (phosphate-free) carp hemoglobin (O) and hemoglobin solutions containing 0.16 mM ATP (∇) and GTP (Δ) measured at 20°C in Tris buffer pH 7.55. Hemoglobin concentration, 0.10 mM total chloride 0.10 mM

in fish erythrocytes to environmental conditions such as water hypoxia. As with diphosphate in mammals, these polyanionic cofactors bind at a specific positively charged site of deoxyhemoglobin depressing its O_2 affinity. Erythrocytes however contain relatively high concentrations of Mg^{2+} which will complex with NTP's impairing their role as regulators of erythrocytic O_2 affinity. This communication reports the effects of ATP and GTP on the O_2 -binding properties of carp hemoglobin and the influence on this of Mg^{2+} in attempts to assess the physiological implications of the hemoglobin-ATP/GTP Mg^{2+} interaction in fish.

ATP and GTP strongly increase the half-saturation O_2 tension (P_{50}) of the phosphate-free hemoglobin (depress O_2 affinity) and raise the Hill coefficient n , which reflects the sigmoid character of the O_2 equilibrium curve (cf. Fig. 1). However, at equal concentrations the GTP effects markedly exceed those of ATP. Double logarithmic plots of $\log P_{50}$ vs. phosphate concentration indicate that binding of both NTP's liberates all bound O_2 from carp erythrocyte hemoglobin.

Mg^{2+} drastically reduces the effects of NTP on O_2 affinity (Fig. 1). However, while increasing Mg^{2+} obliterates the ATP effect, a GTP effect persists. This correlates with previous evidence that GTP binds at an additional site of the hemoglobin perhaps through its greater ability for hydrogen bonding (Weber *et al.* 1975) and indicates that the Mg -GTP complex itself inhibits O_2 binding. Hypoxic- and normoxic-acclimated carps show no significant difference in erythrocytic Mg^{2+} concentrations indicating that the adaptational changes in blood P_{50} are primarily attributable to variation in NTP levels.

The data demonstrate a dominant role for GTP in the *in vivo* regulation of blood P_{50} in fish containing both types of trinucleotides.

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C 20

Influence of EMG and Tension on the Cardiovascular Response to Isometric Muscle Contractions

By B. SCHWY, J. H. MITCHELL, B. SALTIN and P. C. PAYNE. *August Krogh Institute University of Copenhagen, Copenhagen, Denmark.*

It is still an open question to what degree the regulation of the cardiovascular system during isometric muscle contractions is of central and/or peripheral origin. The aim of the present experiments was to investigate the possible effect of central nervous irradiation and peripheral muscle tension on heart rate (HR) and blood pressure (BP) during sustained isometric contraction of the quadriceps muscle. The EMG of vastus lateralis taken as a measure of the central nervous activity. Seven male subjects went through two different

experiments also demonstrate that the active transport mechanism responsible for the post stimulatory reuptake of potassium is very far from being saturated under physiological conditions

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C 19

Functional Interaction between Fish Hemoglobin Erythrocytic Nucleoside Triphosphate and Magnesium

By ROY E WEBER *Department of Zoophysiology University of Aarhus*

Recent studies (Wood & Johansen 1972 Geoghegan & Polubowich 1974 Weber *et al* 1975) have shown that the nucleoside triphosphates (NTP) ATP and GTP (guanosine triphosphate) are allosteric effectors that continuously adapt the O_2 affinity of hemoglobin

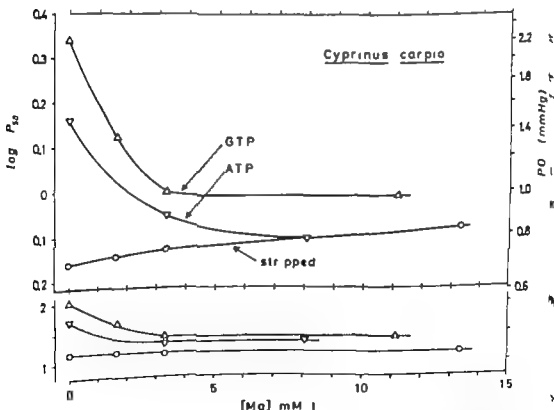


Fig. 1 The effect of magnesium ion on the half-saturation O_2 tension, P_{50} , and Hill's coefficient n of "stripped" (phosphate-free) carp hemoglobin (O) and hemoglobin solutions containing 0.16 mM ATP (V) and GTP (Δ) measured at 20°C in Tris buffer pH 7.55 Hemoglobin concentration 0.10 mM total chloride 0.10 mM

arterial increases of the plasma VIP concentration were noted in the venous effluents of the target organs. No change of arterial VIP concentrations was observed. Furthermore, electrical stimulation of the vagal low threshold fibres, evoking atropine sensitive contractions, produced no increase in gastric venous plasma concentration of VIP. The results obtained thus suggest that VIP may be a neurotransmitter in the gastrointestinal tract mediating specific physiological reflexes and constituting a nervous control system similar to the established cholinergic and adrenergic ones.

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C 22

Effect of Centrally Infused Histamine (HA) and its Analogues in the Conscious Goat

By L. ERIKSSON and L. TUOVISTO *Department of Physiology College of Veterinary Medicine Helsinki and Department of Pharmacology University of Helsinki Finland*

Histamine (HA) a putative neurotransmitter is richly located in the hypothalamus (cf Schwartz 1977). In the guinea pig hypothalamus the increases in the HA content and in the activity of HA-N-methyltransferase occur approximately at the same time during necropsy (Tuovisto 1977) as does the appearance of vasopressin-containing neurosecretory granules (Donev 1970 Silfverman 1975). Therefore the effects of intracerebroventricularly (ICV) infused HA on the water balance are of great interest. In this study HA and its analogues were given ICV to conscious hydrated goats.

Infusion of HA into the third or lateral brain ventricles (10-1000 µg/animal) caused a dose-dependent antidiuretic response in the hydrated goat. The urine osmolality began to rise within 20 min, reached its maximum within 40-120 min and lasted for 90-250 min depending on the dose.

Methylhistamine (2-MH) an H_2 -agonist, caused a weaker but otherwise similar antidiuretic response as HA. Disaprit (300 µg) a specific H_2 -agonist did not change urine osmolality appreciably during the first 3 hours. Subsequently however the osmolality increased. 4-Methylhistamine (4-MH) another H_2 -agonist with some H_1 -activity given at a dose of 300 µg, increased the urine osmolality gradually within a 3-hour period. Infusion of the same agent primarily induced a HA-like antidiuresis. After a partial recovery a secondary bout of antidiuresis ensued.

3-Methylhistamine a metabolite of HA, did not influence the urine osmolality. Other effects of the ICV infusions of HA and its analogues were slight drowsiness, hyperaemia after 3-MH and an elevation of rectal temperature after the H_2 -agonists.

procedures 1) Twenty percent of the maximal voluntary isometric force (%MVC) was maintained for 5 min while HR BP and EMG was followed continuously 2) As experiment 1) except that the EMG was voluntarily kept constant at an initial 20% MVC-level In 1) a continuous rise was seen in HR BP and EMG throughout the 5 min (mean values from 0 to 5 min HR 77.6 to 108.0 BP 130.2/83.4 to 180.3/122.6 mmHg, EMG 112.7 to 218.0 mV) In 2) an increase in HR and BP and a decrease in isometric tension was observed (mean values from 0 to 5 min HR 78.0 to 95.0 BP 133.1/84.9 to 154.3/103.3 mmHg, tension 19.5 to 11.0 %MVC) Looking at the 5 min values it is not possible to deduce whether it is the muscle tension or the EMG which is responsible for the changes in HR and BP since the percentage increase of HR and BP in 2) is about half the increase in 1) while both the EMG and tension in 2) is about half the value of that seen in 1) If however HR and BP is followed during the whole period it can be seen that both parameters increase in both experiments in spite of the fact that muscle tension is constant or decreasing EMG rose continuously in 1) while it was kept constant in 2) Thus neither tension nor EMG is alone responsible for the changes in HR and BP One possible explanation may be deduced from the fact that most of the increase in HR and BP in 2) was seen during the first two min where tension was reduced 50% while a continuous increase in HR and BP was seen in 1) This may indicate that also local metabolic factors may be involved in the cardiovascular regulation during isometric exercise

C 21

Is Vasoactive Intestinal Polypeptide (VIP) a Neurotransmitter in the Gastrointestinal Tract?

By J. FAHRENKRUG, U. HAGLUND, M. JODAL, O. LUNDGREN, L. OLBE and O. B. SCHAFFALITZKY DE MUCKADELL. *Departments of Physiology and Surgery, University of Göteborg, Sweden and Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark.*

The presence of VIP in central and peripheral neurons has been demonstrated with immunohistochemical techniques (Larsson *et al.* 1976) mainly located in synaptosomes (Giachetti *et al.* 1977). These findings suggest that VIP may be a neurotransmitter. Within the gastrointestinal tract the localization of the VIP containing neurons varies. In the stomach they are mainly found in the muscularis layer while in the small and large intestines they are most abundant in the mucosa where they often follow the small blood vessels. Except in the gastric antrum no VIP immunoreactivity was found in the epithelial cells of the alimentary canal. To test the hypothesis of VIP as a neurotransmitter three different nervously controlled effects known to be mediated via non-cholinergic non-adrenergic mechanisms were studied in cats. 1. The gastric relaxation elicited by stimulating electrically the high threshold vagal fibres. 2. The intestinal vasodilatation evoked by mechanically stimulating the intestinal mucosa. 3. The colonic vasodilatation observed upon electrical stimulation of the pelvic nerves. The plasma concentration of VIP before, during and after inducing the nervous effects were determined using the RIA method of Fahrenkrug and Schaffalitzky (1977). Concomitant with the nervously induced effects

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C 24

Role of Cyclic AMP in Oocyte Maturation

By C. EKHOLM, T. HILLENRÖD, C. MAGNUSSON and K. ÅHRÉN *Department of Physiology University of Göteborg Göteborg Sweden*

One of the consequences of the preovulatory surge of luteinizing hormone (LH) is resumption of oocyte meiosis ("oocyte maturation"). The mechanism behind this LH effect in the preovulatory follicle is still unknown. Cyclic AMP has been suggested to play a role in this mechanism since it has been shown (Tsafriri *et al* 1972) that injection of cyclic AMP into preovulatory rat follicles induced oocyte maturation. It has on the other hand, been reported (Cho *et al* 1974) that dibutyryl cyclic AMP (dbcAMP) inhibited the spontaneous maturation of isolated mouse oocytes. The aim of the present study has been to further elucidate the possible role of cyclic AMP in this context.

Preovulatory follicles were obtained from immature rats pretreated with a low dose of pregnant mare's serum gonadotrophin two days earlier. Oocytes were dissected from the follicles and oxygen consumption of the isolated oocytes was measured according to Hultén (1974). Respiration was measured in oocytes directly isolated from the follicles and in oocytes first cultured in a modified Brinster phosphate buffer (Magnusson *et al* 1977) prior to determination of oxygen consumption. The oocytes were examined by interference contrast microscopy using germinal vesicle breakdown (GVB) as the first sign of oocyte maturation. The cultured oocytes, which had resumed meiosis (all with GVB), showed higher oxygen consumption than the dictyate oocytes taken directly from the preovulatory follicles. When cyclic AMP (10 mM) or dbcAMP (1 mM) was added to the culture medium GVB was prevented and the rise in oxygen consumption was abolished. The effect of cyclic AMP was reversible since GVB was present after another 4 hrs culture period in nucleotide free medium.

In a second series of experiments isolated preovulatory follicles were treated in a double incubation procedure starting with 2 hrs incubation with dbcAMP (10 mM) followed, after a 30 min wash period by 4 hrs incubation with or without dbcAMP. For one group of follicles plain medium was used for both incubation periods. The last group of follicles showed a low rate of GVB (17%) while presence of dbcAMP throughout the whole incubation period completely abolished GVB. Transfer from dbcAMP-containing to plain medium, on the other hand, enabled 70% GVB. The inhibiting effect of dbcAMP on oocyte maturation was thus not only reversible but dbcAMP if present only in the first incubation period exerted a stimulatory effect on GVB.

A possible interpretation of our own data as well as other experiments is 1) that an increased level of cyclic AMP in or around the oocyte itself is not involved in the mechanism

These results confirm the earlier observation from this and other laboratories of the antidiuretic effect of HA in various species (*cf* Schwartz 1977). Our studies with H_1 - and H_2 -agonists speak for the role of H -receptors in the HA induced antidiuresis in the hydrated goat. The delayed antidiuretic effect of the H_2 -agonists needs further study. In part it may result from the increased body temperature.

The gifts of histamine analogues from the Smith Kline and French Laboratories are gratefully acknowledged.

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C 23

Dopamine- β -hydroxylase and Calcium in Adrenaline- and Noradrenaline-rich Granules Bovine Adrenal Medulla

By G. BOLSTAD, G. SERCK HANSEN and K. B. HELLE

The discharge of soluble dopamine- β -hydroxylase (D β H) activity in concert with catecholamines (CA) has been taken as a measure of the exocytosis of the catecholamine hormones in response to neurochemical and electrical stimulation of the adrenomedullary cells (Viveros, Arqueros and Kirshner 1968). Acetylcholine (100 μ M) stimulates however the release of less enzyme activity/ μ mole CA from the perfused bovine adrenal than apparent in the core of the isolated chromaffin granules (Helle and Serck Hansen 1977). Furthermore stimulus related D β H activity correlates in a positive manner with NA but not with A. These observations might suggest exocytotic release from a subpopulation of granules with a composition different from that of the total population of chromaffin granules.

The chromaffin granules can be partially separated into A- and NA-rich fractions by sedimentation in hypertonic gradients ranging from 1.6-2.0 M sucrose (Eade 1958). Granules have now been sedimented into 2.7 M sucrose. These granules contained 66% of their amines as NA and were considerably richer in total amines (5.05 ± 0.86 μ moles CA/mg protein, mean \pm S.E., $n=10$) than the A-rich granules sedimenting in the 1.6 M sucrose layer (69% A, 4.4 ± 0.29 μ moles CA/mg protein, mean \pm S.E., $n=10$).

The NA-rich granules are considerably lower in their ratio of soluble enzyme activity per μ moles CA than the A-rich granules and contain twice the amount of calcium per unit weight.

It remains to be established to what extent these NA-rich granules participate in the exocytotic release of the marker enzyme.

RI-4 cells could have double-peaked spectral sensitivities and only the R7 single-UV sensitivity

In this study we measured the spectral sensitivity of individual receptors and second order neurons in retina of fly *Calliphora*. Intracellular recordings were carried out with capillary microelectrodes filled with Procion Yellow solution. In total 23 receptors and second order neurons were recorded and identified. Three groups of receptors found: cells with high UV (353 nm) and very low visible region sensitivity (1 0.03)

cells with high UV and low visible region sensitivity (1 0.85) and cells with low UV but visible region sensitivity (0.65 1). Two major groups of second order neurons were found: cells with high UV and relatively low visible region sensitivity (1 0.35) and cells with high sensitivity in both regions (1 0.94). The sensitivity recordings were made in non-saturated region of the intensity-response function. The spectral sensitivity of the cell was found to be attributed to the same morphological structure in different preparations.

These preliminary results give a rise for assumption that a revision of morphological classification is necessary according to the cell function.

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C 26

Influence of Nerve-muscle Activity on the Elimination of Synapses in Neonatal Rat Muscle

By N. THOMPSON, D. KUFFLER and J. K. S. JANSEN. *From the Institute of Physiology, University of Oslo, Norway*

Elimination of the rat soleus muscle changes in the first two weeks after birth from a state where each muscle fiber is innervated by an average of five separate nerve fibers (polyneuronal innervation) to the adult state in which each muscle fiber is singly innervated (Rafferty 1970). Although this elimination of innervation is known to occur because a small number of motor neurons each reduce the number of muscle fibers they innervate (Brown et al. 1976) little is understood about its cause.

To block the conduction of nerve impulses in the sciatic nerve we have investigated whether the functional activity of the synapses in neonatal muscles is required for the elimination of polyneuronal innervation. Nerve blocks were accomplished by insertion into the sciatic nerve of a plastic pellet (Sanger et al. 1976) containing tetrodotoxin (TTX) and which blocks nerve impulses. Using these TTX pellets we have obtained total elimination of impulses in the soleus motor axons for up to 9 days.

We have found that such blocks initiated in 9 or 10-day old animals (just prior to the onset of the first elimination of polyneuronal innervation) result in a rapid

of oocyte maturation 2) that other LH-induced changes in the granulosa cells are responsible for oocyte maturation and 3) that LH induced increases in granulosa cell cyclic AMP levels thus indirectly are of importance for resumption of oocyte maturation.

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C 25

Colour Sensitivity of Fly's Retina

By M. JÄRVILEHTO, J. MORING and KRISTINA MORING *Department of Physiology University of Oulu, Finland*

Behavioral studies have shown that many insects possess a colour vision (v. Frisch 1965). In the fly's retina at least two visual pigments with different absorption maxima have been found by using microspectrophotometry. Electrophysiological methods revealed three major groups of receptor cells with different sensitivity maxima, all having a second maximum in the UV region. Besides those double-peaked sensitivities, cells with single-peaked sensitivity in UV have been reported by recent investigators. Based on microspectrophotometry and genetical selection experiments with *Drosophila*, it has been concluded

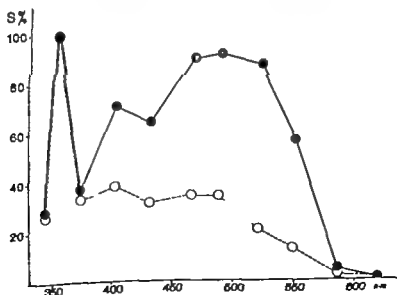


Fig. 1 The relative spectral sensitivity of second order neurons. ● UV VIS cells ○ UV cells.

for all R1-6 cells could have double-peaked spectral sensitivities and only the R7 single peaked UV sensitivity

In this study we measured the spectral sensitivity of individual receptors and second order neurons in retina of fly *Calliphora*. Intracellular recordings were carried out with glass capillary microelectrodes filled with Procion Yellow solution. In total 23 receptors and 25 second order neurons were recorded and identified. Three groups of receptors were found: cells with high UV (353 nm) and very low visible region sensitivity (1-0.03) cells with high UV and low visible region sensitivity (1-0.85) and cells with low UV but high visible region sensitivity (0.65-1). Two major groups of second order neurons were found: cells with high UV and relatively low visible region sensitivity (1-0.35) and cells with high sensitivity in both regions (1-0.94). The sensitivity recordings were made in non-stimulated region of the intensity-response function. The spectral sensitivity of the cell was not found to be attributed to the same morphological structure in different preparations. These preliminary results give a rise for assumption that a revision of morphological classification is necessary according to the cell function.

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C 26

The Influence of Nerve-muscle Activity on the Elimination of Synapses in Neonatal Rat Soleus Muscle

By W. THOMPSON, D. KUTFLER and J. K. S. JANSEN *From the Institute of Physiology University of Oslo, Norway*

The innervation of the rat soleus muscle changes in the first two weeks after birth from a state where each muscle fiber is innervated by an average of five separate nerve fibers (polyneuronal innervation) to the adult state in which each muscle fiber is singly innervated (Redfern 1970). Although this elimination of innervation is known to occur because a constant number of motor neurons each reduce the number of muscle fibers they innervate (Brown *et al.* 1976) little is understood about its cause.

By blocking the conduction of nerve impulses in the sciatic nerve we have investigated whether the functional activity of the synapses in neonatal muscles is required for the elimination of polyneuronal innervation. Nerve blocks were accomplished by insertion into the sciatic nerve of a plastic pellet (Sanger *et al.* 1976) containing tetrodotoxin (TTX) and which blocks nerve impulses. Using these TTX pellets we have obtained total blocks of impulses in the soleus motor axons for up to 9 days.

We have found that such blocks initiated in 9 or 10-day old animals (just prior to the appearance of the first singly innervated fibers) do not prevent the appearance of singly

of oocyte maturation 2) that other LH-induced changes in the granulosa cells are responsible for oocyte maturation and 3) that LH-induced increases in granulosa cell cyclic AMP levels thus indirectly are of importance for resumption of oocyte maturation

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C 25

Colour Sensitivity of Fly's Retina

By M JÄRVILEHTO, J MORING and KRISTINA MORING, Department of Physiology, University of Oulu, Finland

Behavioral studies have shown that many insects possess a colour vision (v. Frisch 1965). In the fly's retina at least two visual pigments with different absorption maxima have been found by using microspectrophotometry. Electrophysiological methods revealed three major groups of receptor cells with different sensitivity maxima, all having a second maximum in the UV region. Besides those double-peaked sensitivities, cells with single-peaked sensitivity in UV have been reported by recent investigators. Based on microspectrophotometry and genetical selection experiments with *Drosophila*, it has been concluded

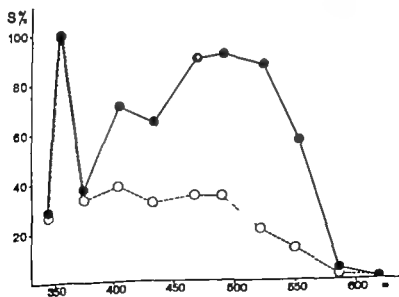


Fig. 1 The relative spectral sensitivity of second order neurons. ● UV-VIS cells ○ UV cells.

were used. In both positions the depolarizing effect seemed to be more effective than the hyperpolarization in diminishing the IPSP amplitude, membrane resistance as well as the firing frequency. The hyperpolarizing, but not the depolarizing effect of GABA, could be reversed by passing currents across the cell membrane. The reversal potential for GABA applied at the soma was the same as for the IPSP. Addition of 10^{-6} mM bicuculline reduced both GABA response types and changed the orthodromic response from a single spike to a long burst of discharges.

The results support the idea that the hyperpolarizing basket cell inhibition in the hippocampus is mediated by release of GABA on the somata of pyramidal cells (Curtis *et al.* 1970). In addition, there may be interneurons that release GABA on dendrites, causing localized inhibition by a conductance shunt mechanism. This would fit with the accumulation of glutamate decarboxylase both near the soma and at certain dendritic positions (Fossum and Storm-Mathisen 1969).

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C 28

Delayed Drinking Induced by Electrical Stimulation of the Medial Forebrain

By M. RYNDGREN *Department of Physiology I Karolinska Institutet Stockholm S edn*

Strictly Mauthner-bound drinking was previously induced by unipolar electrical stimulation within the perifornical region of the hypothalamus in the goat (Anderson and McCann 1970). However the integrated results of later studies in the same species imply that cerebral sodium sensitive receptors regulating water intake predominantly are located more medially close to the anterior border of the 3rd ventricle (cf Anderson 1977). Therefore, it was of interest to include this area in a field of bipolar electrical stimulation in the ongoing work.

10 goats were used for expts over a period of 8 weeks. Each animal had a pair of stainless steel electrodes permanently implanted bilaterally into the medial forebrain (3 mm apart and with 3 mm uninsulated tips) at the level of the anterior commissure. Modified Hess technique was employed with stimulus parameters: 0.4-3 mA, 50 c/s pulse duration 1.5 ms stimulation periods 1-10 min.

One goat invariably responded with drinking to current strength of 1 mA or higher. Drinking generally started 20-30 s after stimulation, and 0.5 to 2.5 liters of water were drunk. The water consumption was roughly proportional to the amount of current applied. When the animal was not allowed to take significant amounts of water after the stimulation obvious urge to drink remained for 8 to 10 min. Stimulations up to the strength of

Innervated fibers in the muscle although the rate of their appearance is reduced. On the other hand 5-6 days after the initiation of nerve block a reversal of synapse elimination was noted with the reappearance of more polyneuronally innervated fibers.

We conclude that the functioning of the synapses (or the muscle activity associated with it) is not required for elimination to occur. Extended nerve block, however, is able to reverse elimination and produce nerve sprouting.

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C 27

Two Separate Effects of GABA on Hippocampal Pyramidal Cells *in vitro*

By I. A. LANGMOEN, P. ANDERSEN, L. GJERSTAD, A. MOSFELDT LAURSEN and T. GANES *Institute of Neurophysiology, University of Oslo*

The aim of the present study was to examine the mode of action of gamma-aminobutyric acid (GABA) on hippocampal pyramidal cells. Intracellular recordings were taken from CA1 pyramids in hippocampal slices *in vitro* (Skrede and Westgaard 1971). Two different effects of iontophoretically applied GABA were observed when applied close to the soma. GABA hyperpolarized the cell, whereas it depolarized the neurone when delivered in the dendritic tree. In both cases there were potential changes associated with an increase of membrane conductance, reduced IPSP amplitude and cessation of spontaneous and evoked discharges. (Fig. 1)

The depolarization which followed ejection of GABA among the dendrites changed to a hyperpolarization lasting for the duration of the ejection. The hyperpolarization following soma ejection was followed by a depolarization when stronger GABA ejection currents

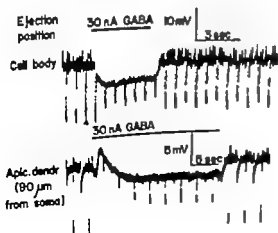


Fig. 1. Ejection of GABA at the cell body (upper half) and in the apical dendritic layer (lower part). Both effects were accompanied with a fall in membrane resistance (Δ). 30 nA ejecting current, \square nA backing current. IPSP (\bullet).

DEMONSTRATIONS

D 1

RV/TLC Ratios Measured with ^{133}Xe -radiospirometry and He-dilution Methods

By K. KAUPPINEN-WALIN, H. HOLLI, A. R. A. SOVIÄRVI and A. UUSITALO
*Department of Biomedical Sciences, University of Tampere and Department of Clinical
 Physiology, Tampere Central Hospital, Tampere, Finland*

^{133}Xe -radiospirometry has been shown to be a clinically suitable method for acquiring information on the distribution of ventilation, the perfusion and the ventilation/perfusion ratio of the lungs. The validity of a multidetector type radiospirometer for acquiring quantitative data of lung volumes has, however, been discussed. But regardless of whether the volumes *per se* are valid, their ratios should be no different from the ratios conventionally determined. And the residual quota is quite useful in evaluating the degree of a patient's hyperinflation.

To establish the reliability of radiospirometrically determined residual quotas we measured the RV/TLC of 30 healthy people with a multidetector type ^{133}Xe -radiospirometer on one hand and with a He-dilution in closed circuit on the other hand. The methods were similar in that they both employ a foreign gas for an indicator to calculate the volumes RV and TLC.

The subjects comprised 13 young people, 19 to 31 years old, seven women and six men and 17 old people, 65 to 70 years old, nine women and eight men. The RV/TLC ratio was determined for each subject with both methods, both in sitting and in supine positions.

The radiospirometric results on RV/TLC determinations showed no differences between individual first and second determinations. Neither were any significant differences between sitting and supine RV/TLC ratios exposed. Thus, we analyzed the data obtained with the first measurement by both methods as done in clinical routine with the He-dilution method. It was the first in the sitting position and with radiospirometry the first in the supine position.

Statistical analysis (paired observation t-test) showed that the residual quota determined with the two methods, He-dilution and ^{133}Xe -radiospirometry, was essentially the same ($P > 0.05$).

Thus it seems that RV/TLC ratios produced automatically along with routine radiospirometric lung function examinations with the multidetector type ^{133}Xe -radiospirometer are valid data for estimation of the degree of a patient's possible hyperinflation.

2 mA did not induce any other obvious behavioural effects. On repeated stimulations within two hrs there was a gradual attenuation of the drinking response. Likewise pre-stimulatory hydration of the goat markedly reduced its sensitivity to stimulation. A similar but less pronounced and consistent dipsogenic effect was obtained in the second goat. Here drinking commenced 1 to 2 min after discontinuation of the electrical stimulation.

The delayed drinking induced by electrical stimulation in the present experiments is different from the strictly stimulus-bound drinking earlier obtained in response to unilateral hypothalamic stimulation (Andersson and McCann 1955). An explanation of the difference might be that the previous unilateral stimulations mainly affected immediately responding efferent pathways from "thirst-receptors" located close to the ventricular wall and that these receptors for some reason react with post-stimulatory excitation to the application of electrical impulses. The present field stimulation may gradually have induced a local redistribution of Na⁺ sufficient to activate the thirst mechanism in the post-stimulatory period.

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no less important in work on smooth muscle than on striated muscle. The apparatus described here is used with smooth muscle strips of 1-4 mg weight and 3-7 mm length developing forces of 6-30 mN. Change from isometric to isotonic conditions can be accomplished in less than 2 ms and length oscillations after the step usually do not extend to more than 10 ms, for small force steps considerably less. A 20 mm long lever made of stainless steel wire, is mounted on micro ball bearings. It is tapered from a diameter of 7 mm near the fulcrum to 0.2 mm near the tip. Afterload is provided by an adjustable spring acting on the lever close to the fulcrum. A photoelectric displacement transducer (sensitivity 10 V/mm) is influenced by a small vane mounted on the lever axis. The physical mass of the moving parts is 8 mg. The lever is hanging vertically with its tip secured in the muscle bath. Two independently adjustable stops acting in the forward and backward directions, make contact with the lever in the solution 4 mm above the site of muscle attachment, thereby eliminating adhesion. The stops can be released by electromagnetic relays, and are checked by micrometers permitting either free isotonic lenses or controlled length steps. Due to its small dimensions the lever will bend somewhat when an afterload force (up to 30 mN) is applied causing a shift in baseline of the displacement transducer. A force of the same magnitude applied at the muscle attachment will, however, cause only negligible deformation ($0.2 \mu\text{m}/\text{mN}$) due to the position of the stops. Therefore withdrawal of the stops as in a quick release will not appreciably alter the strain on the lever. The muscle is mounted horizontally with its ends wrapped into small pieces of aluminum foil, one of which is hooked on to the sharpened tip of the lever and the other in a similar tip connected to a capacitance force transducer (sensitivity 0.1 $\mu\text{m}/\text{mN}$, compliance $0.2 \mu\text{m}/\text{mN}$, natural frequency 620 Hz). The bath is a thermoregulated perspex trough perfused with oxygenated solution. Contraction of the muscle is induced by means of electrical stimulation, K^+ -high solution or drugs. The whole mechanical arrangement is rigidly mounted on a heavy iron plate.

D 4

Catecholamine Content in Blood Plasma, Heart and Intercostal Arteries of the African Lungfish

By T. ABRAHAMSSON, S. HOLMÖREN, S. NILSSON and K. PETTERSSON, Department of Zoophysiology, University of Göteborg, Sweden

The chromaffin tissue which corresponds to the catecholamine secreting adrenal medulla in the mammals, shows a variable arrangement among the lower vertebrates. In the cyclopterus chromaffin cells are found in large quantities within the heart and also in the walls of the cardinal veins. The elasmobranchs have chromaffin cell groups arranged segmentally in connection with the sympathetic ganglia and the chromaffin tissue in the elasmobranchs is mainly within the anterior part of the kidney (head kidney) often lining the walls of the posterior cardinal veins.

The chromaffin tissue of the African lungfish, *Protopterus* (first demonstrated by Glazman (1906) and Holmes (1940)) is located in the walls of the intercostal branches of the

D 2

Tissue Osmolality in the Papillae of the Cat's Tongue

By DAN AXEL HALLBACK, MATS JODAL and OVE LUNDGREN *Department of Physiology, University of Göteborg, Sweden*

The tongue papillae have a vascular arrangement which resembles that of the intestinal villi i.e. they are supplied by an arterial vessel that runs centrally to the tip where it branches into a dense capillary network. The two blood flows running in close approximation and in opposite direction to each other constitutes the prerequisite for the presence of a countercurrent exchanger in the papillae. Such a mechanism may act by concentrating absorbed food substances in the papillary tips and may thereby be of importance for the sensation of taste.

To test this hypothesis the tissue osmolality at different levels of the papillary core was determined with a cryoscopic method described by Jodal *et al* (1977). The surface of cat's tongue was flushed for at least 20 min with isotonic Krebs solutions containing different amounts of sodium and glucose. The tongues were then momentarily frozen in isopentane precooled in liquid nitrogen and cut obliquely in 15 μ m thick sections so as to give cross-sections at different levels of the papillae. By stepwise increasing the tissue temperature the tissue osmolality could be determined as a freezing point depression in the papillary core.

Both filiform and fungiform papillae showed a marked osmolar gradient from tip to base of the papillae when flushed with an isotonic Krebs glucose solution, the tip values being about 600–800 mOsm/kg H_2O . Two different types of filiform papillae were studied in more detail. In one type, being large and horny, a significant decrease in tip osmolality occurred when glucose was replaced by mannitol. Exchanging also the sodium ions with choline reduced the tip osmolality to the same value as found in *in vitro* condition i.e. about 400 mOsm/kg H_2O . In the other type of filiform papillae no change in the osmotic gradient was seen with the replacement of either sodium and/or glucose. The findings support the hypothesis of a presence of a countercurrent exchanger in the tongue papillae and they also provide evidence for different electrolyte transport mechanisms in different papillae.

Reference

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D 3

An Apparatus for Mechanical Experiments on Isolated Smooth Muscle

By L. SJÖLIN, P. HELLSTRAND and B. CLEMENTZ. *Department of Physiology and Biophysics, University of Lund, Sweden*

Isotonic quick release and quick stretch experiments on muscle require an experimental design to minimize inertial forces if disturbing length oscillations are to be avoided. This

is no less important in work on smooth muscle than on striated muscle. The apparatus described here is used with smooth muscle strips of 1-4 mg weight and 3-7 mm length developing forces of 6-30 mN. Change from isometric to isotonic conditions can be accomplished in less than 1 ms and length oscillations after the step usually do not extend over more than 10 ms, for small force steps considerably less. A 20 mm long lever made from stainless steel wire is mounted on micro ball bearings. It is tapered from a diameter of 0.7 mm near the fulcrum to 0.2 mm near the tip. Afterload is provided by an adjustable plate spring acting on the lever close to the fulcrum. A photoelectric displacement transducer (sensitivity 10 V/mm) is influenced by a small vane mounted on the lever axis. The equivalent mass of the moving parts is 8 mg. The lever is hanging vertically with its tip immersed in the muscle bath. Two independently adjustable stops, acting in the forward and backward directions, make contact with the lever in the solution 1.4 mm above the point of muscle attachment, thereby eliminating adhesion. The stops can be released by electromagnetic relays and are checked by micrometers permitting either free isotonic releases or controlled length steps. Due to its small dimensions the lever will bend somewhat when an afterload force (up to 30 mN) is applied, causing a shift in baseline of the displacement transducer. A force of the same magnitude applied at the muscle attachment will, however, cause only negligible deformation ($0.1 \mu\text{m/mN}$) due to the position of the stops. Therefore, withdrawal of the stops as in a quick release will not appreciably alter the strain on the lever. The muscle is mounted horizontally with its ends wrapped into small pieces of aluminum foil, one of which is hooked on to the sharpened tip of the lever and the other to a similar tip connected to a capacitance force transducer (sensitivity 0.1 V/mN, compliance $0.2 \mu\text{m/mN}$, natural frequency 630 Hz). The bath is a thermoregulated 1 ml perspex trough perfused with oxygenated solution. Contraction of the muscle is induced by means of electrical stimulation, K^+ -high solution or drugs. The whole mechanical arrangement is rigidly mounted on a heavy iron plate.

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D 3

An Apparatus for Mechanical Experiments on Isolated Smooth Muscle

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Isotonic quick release and quick stretch experiments on muscle require an experimental design to minimize inertial forces if disturbing length oscillations are to be avoided. This

8. 11). The present study reports upon the nature of the receptors responsible for these acts.

Rabbits were anesthetized with chloralose and urethan and placed on their back with head fixed to the table. Changes in position of the left maxillary incisor were measured by ultrasonic technique as before.

Responses of the tooth to bilateral stimulation of the cut cervical sympathetic nerves (1 ms, 2-30 Hz) were reduced by i.v. phenoxybenzamine (above 3 mg/kg) largely blocked by phentolamine (2-3 mg/kg). Remaining responses disappeared after infusion of propranolol (0.3-1.5 mg/kg). When not preceded by phenoxybenzamine propranolol blocked the responses to nerve stimulation. Atropine was without effect.

Propranolol induced a rapid extrusion, of a magnitude greatly exceeding that resulting on mechanically evoked rises in arterial or venous pressure (aortic balloon inflation, occlusion of jugular veins). Isoprenaline (0.3-0.6 mg/kg) reversed or prevented this movement.

These results can be explained by the existence of a double adrenergic control of vessels regulating the axial position of the tooth, sympathetically innervated alpha-receptors effecting constriction of pre-capillary vessels and beta-receptors effecting dilatation of post-capillary resistance vessels. The latter type of receptors must be activated mainly by humoral agents. Changes in position of the tooth presumably reflected alterations in capillary blood volume or tissue pressure. The recently demonstrated importance of pressure and tone in periodontal vessels for the position of the rabbit's incisor (Myhre, L. I. R. Preus and H. Aars, *Acta physiol scand* 1977 this meeting) suggests that the presently observed receptors were located to the periodontal rather than the periapical or alveolar vascular bed.

D 6

Schemata Controlling Mobility and Position of the Rabbit's Tooth

By L. MYHRE, H. R. PREUS and H. AARS. *Department of Physiology and Biochemistry Dental Faculty University of Oslo Oslo Norway*

Teeth are known to be firmly "kept in place" —yet they move when subjected to loads exceeding a few grams. This dynamic resistive property of the tooth has been ascribed to the collagenous fibres and the vasculature of the periodontium, the pulpal tissue pressure, or to the periapical tissue. We have investigated the mobility of the rabbit's incisor and the localization of the tissue determining this mobility by recording the movements of the incisor in response to axial loads before and after the tooth had been cut close to the apex. We also studied the movements induced by vasoactive drugs and changes in systemic arterial blood pressure.

The rabbits were anesthetized with chloralose and urethan, and placed on their back with the head fixed to the table. Positional changes of the left maxillary incisor were recorded by ultrasound transit time technique: one crystal was fastened to the tooth the other to the skull by a metal bridge. Loads of 5-20 g and 20 s duration were applied by

dorsal aorta. A sympathetic innervation of this tissue was also described by Holmes (1950)

As part of a comparative study of adrenergic mechanisms in lower vertebrates conducted in this laboratory a preliminary study has been carried out in a few specimens of the African lungfish *Protopterus aethiopicus*

Samples of blood plasma, the heart and the intercostal arteries were assayed spectrofluorimetrically for adrenaline and noradrenaline. The concentrations of adrenaline and noradrenaline were very similar in the plasma of control animals (2.3 ± 0.8 and 2.4 ± 1.1 $\mu\text{g}/100$ ml plasma respectively means \pm S.E. $n=6$) but after "stress" induced by chasing the animal around its tank for about 15 min the noradrenaline concentration was increased more than 20-fold while the adrenaline level was increased only 4-fold (means of two animals only)

Large quantities of adrenaline and especially noradrenaline (4.2 ± 1.1 and 70.8 ± 17.3 $\mu\text{g/g}$ tissue respectively means \pm S.E. $n=6$) were found in the heart. These high levels compare with the situation in cyclostomes (Augustinsson *et al* 1956 Euler and Fänge 1961) Much lower quantities of the catecholamines were found in the intercostal arteries in this initial study (adrenaline 0.3 ± 0.1 $\mu\text{g/g}$, noradrenaline <0.1 $\mu\text{g/g}$ tissue means \pm S.E. $n=8$)

In conclusion the preliminary data so far obtained indicate a storage of large quantities of especially noradrenaline in the heart, a situation resembling that in cyclostomes. We have been unable with the present method to find but smaller quantities of catecholamines in the intercostal arteries where chromaffin tissue has been described by Giacomini (1906) and Holmes (1950)

Further extensive work is needed to clarify the correlation between the high noradrenaline levels in the heart and in the plasma after "stress" and the nature of the previously described chromaffin tissue in the intercostal arteries

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D 5

Adrenergic Receptors in Periodontal Vessels

By H. AARS, Department of Physiology and Biochemistry Dental Faculty University of Oslo Oslo Norway

The position of the rabbit's incisor is tonically controlled by sympathetic nerves cutting the cervical sympathetic nerves makes the tooth move out of its socket (extrusion) while stimulation of the nerves causes intrusion (Aars H. *Acta physiol scand* 1976. Suppl. 1)

ocytes that had started to mature showed an increased oxygen consumption as compared to the dictyate oocytes. Following culture in the absence of hormone the cumuli showed a transient decrease in oxygen consumption at 1 hr of culture when LH was present in the medium this decrease was continuous. Oocytes resumed meiosis "spontaneously" after 1 to 2 hrs in culture. At the same time an increase in oxygen consumption was recorded.

The present study shows that there is a close correlation in time between the metabolic changes in the oocyte and in the cumulus cells both following *in vivo* and *in vitro* stimulation, indicating that changes in energy metabolism in these two follicular cell types might be of importance for the resumption of meiosis. The lag period observed following stimulation with LH *in vivo* as compared to the rapid response during *in vitro* culture might reflect that LH *in vivo* has to counteract an inhibitory influence which is rapidly removed by isolation but needs longer time for inactivation *in vitro*.

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D 8

Differences between Near-threshold Membrane Currents in Slowly and Fast Adapting Lobster Stretch Receptor Neurons

By W. GRAMPP Department of Physiology and Biophysics University of Lund
 Sweden

Near-threshold membrane currents were determined in slowly and fast adapting lobster stretch receptor neurones in an attempt to explain these cells' differing firing characteristics. In this connection major dissimilarities in the presence and control of these currents were found in the two cell types. Some of these dissimilarities are illustrated in Fig. 1 showing the steady state voltage dependence of threshold and subthreshold membrane currents in the slowly and fast adapting cell.

Further differences were related to the facts (1) that a transiently activatable 4-aminopyridine sensitive K current was in near-threshold potential regions present mainly in the fast adapting cell and (2) that a fraction of the TTX sensitive Na current associable with the generation of membrane noise (Sjölin and Grampp 1975) was observable only in the slowly adapting cell.

In steady state the voltage dependence of the various membrane currents proved to be of such a kind that in the slowly adapting cell it gave rise to the formation of a negative slope of the relationship between membrane polarisation and total ionic membrane current in suprathreshold potential regions. In this way conditions were created making possible

means of a rod connected to an electromagnet. Blood pressure was measured with a catheter in the brachial artery.

The amplitude of inward movements (intrusion) increased with increasing loads. Intrusion and extrusion after release of the load showed an initial rapid phase followed by a slower, less pronounced further movement. Neither amplitude nor pattern of movements were altered after sectioning of the tooth or during the first two days after the animal had been killed. We therefore conclude that the mobility of this continuously erupting tooth was determined by the properties of the periodontal collagenous fibres.

A rise of blood pressure produced by inflation of an intra-aortic balloon resulted in extrusion of the tooth. Norepinephrine i.v. raised arterial pressure but led to intrusion of the tooth, whereas i.v. papaverine caused a slight extrusion if pressure was maintained by means of the balloon. Since the pressure-induced extrusion remained unchanged after the tooth had been cut but was greatly diminished by infiltration of the periodontium with norepinephrine, the responses of the tooth to alterations in pressure and vascular tone most likely were due to changes in vascular volume or tissue pressure in the periodontium.

D 7

Further Studies on the Metabolism of the Preovulatory Rat Oocyte and Cumulus Oophorus

By C. MAGNUSSON and T. HILLENSJÖ, *Department of Physiology, University of Göteborg, Sweden*

The preovulatory surge of LH induces the resumption of oocyte meiosis (oocyte maturation) as well as morphological changes in the cumulus cells. The meiotic process proceeds from the dictyate stage up to metaphase II close to ovulation. The mechanism for the LH effect on oocyte maturation is not yet clarified and neither is the primary target cell for this action localized. It has earlier been shown that LH acutely stimulates aerobic glycolysis of the whole preovulatory follicle (Nilsson 1974) and decreases the oxygen consumption of the cumulus cells (Dekel *et al.* 1976). In a recent study it was furthermore shown that the oxygen consumption of the oocyte itself increases following the onset of oocyte maturation (Magnusson *et al.* 1977).

The aim of this study was to analyze more closely the temporal relationship between the respiratory changes in the oocyte itself and in the cumulus cells. Oocytes and cumuli were obtained from immature rats pre-treated with a low dose of pregnant mare's serum gonadotrophin two days earlier. The isolated cells were placed in a minute gas-tight chamber containing medium with hemoglobin as an indicator of oxygen tension and the oxygen consumption by the cells recorded according to Hultborn (1974). Oocyte maturation was induced *in vivo* by i.p. injection of 10 µg NIH LH-S18. Oocyte maturation was also induced artificially by isolating the oocytes from the follicles into culture ("spontaneous maturation").

Following the LH injection, oxygen consumption of the isolated cumulus remained unchanged for 3 hrs. Four hours after the injection a significant decrease was seen. In the oocytes the first significant change occurred 3 to 4 hrs after the LH injection. At this time

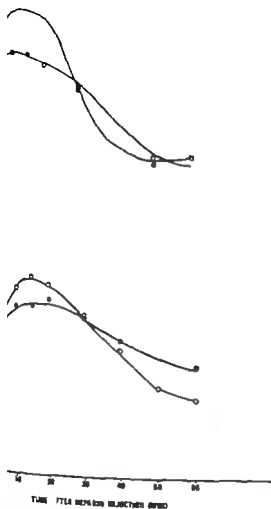


Fig. 1 Total lipolytic activity of postheparin plasma in two test subjects. Subject A (circles): male, age 29 years, height 186 cm and weight 74 kg; B (squares): male, 34 years, 166 cm, 104 kg. Open symbols represent the activity at rest and the closed ones during physical exercise.

familial hypertriglyceridemia but the role of physical exercise as a possible effect of triglyceride lipases has deserved less attention. In the present study the enzymatic activity was determined using ^3H -triolein as substrate described by Huttunen *et al* (1975) with slight modifications. For the selective measurement of hepatic and extra-hepatic (lipoprotein) lipase the inhibition of lipoprotein lipase by 1.0 mol/l NaCl was utilized. No specific antiserum was used. In the experiments a cannula was inserted into an antecubital vein of the subjects. They pedaled an electronically braked bicycle ergometer against light load for 5 min. After heparin injection the load was increased to represent about 45 % of the subject's maximal oxygen

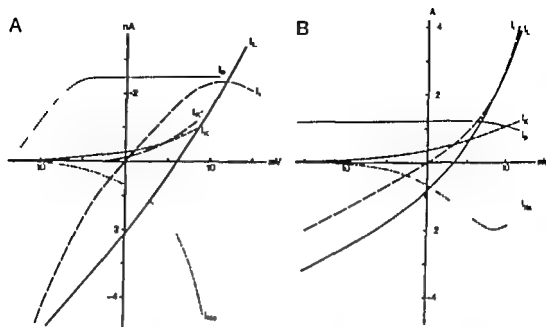


Fig. 1 Steady state voltage dependence of membrane currents in the slowly (A) and fast (B) adapting lobster stretch receptor neurone. I_T , total ionic current; I_{Na} , TTX sensitive Na current; I_K , TEA sensitive K current; I_L , Co sensitive TEA resistant K current; I_p , pump current. Resting potential (corresponding to origo) at -65 mV in A and -67 mV in B.

the generation of maintained repetitive impulse activity in response to long-lasting suprathreshold stimuli. In the fast adapting cell the slope of the steady state relationship between membrane polarisation and total ionic membrane current was found to be positive at all potentials. This may explain the cell's inability to maintain repetitive impulse firing during prolonged suprathreshold stimuli.

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D 9

Triglyceride Lipase Activities of Human Postheparin Plasma in Obese and Non-obese Subjects in Physical Exercise

By J. MARNEMI, P. PELTONEN, I. VUORI and H. HIETANEN. *The Rehabilitation Examinations Centre of the Social Insurance Institution, 20720 Turku 72 and Department of Physiology, University of Kuopio, 70100 Kuopio 10, Finland*

Heparin injection is known to release lipolytic enzymes into human circulation (Korn 1959). These enzymes originate partly from the capillary endothelium and partly from the liver (Huttunen *et al.* 1975). The enzymes have been studied especially in obesity and in

from the three stains were then compared. Based on the NADH D stain 67% (47-87) of the fibres were classified as FTa and FTb in agreement with the ATPase (pH=4.6) stain. The corresponding result from the α GP D stain was 41% (11-81). Only 28% (11-59) were classified identically in all three histochemical stainings. The most inconsistent results were obtained from the orienteers where the number of FTb fibres was overestimated from the NADH D and α GP-D stains.

In conclusion: human skeletal muscle is composed of 2 major fibre types but subgroups definitely do exist. When using histochemical stainings for classification of FTa and FTb fibres it is, however, difficult to ascertain any other way than using an ATPase (pH=4.6) stain.

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D 11

Effect of Hypoxia *Vivo* on the Concentrations of Certain Metabolites in Rat Liver Cytosol and Mitochondria

By V. L. KRONLA and L. HASSINEN, *Departments of Physiology and Medical Biochemistry, University of Oulu, Finland*

The tissue concentrations of certain metabolites were measured after varying periods of hypoxia in order to obtain a better understanding of the reactions of the energy metabolism under such conditions. When the oxygen supply is inadequate the cellular redox state can be expected to become more reduced. The indirect metabolite technique enabled the free NADH/NAD ratio to be calculated from the ratio of the measured concentrations of the oxidized and reduced substrates of a compartmentalized NAD-linked dehydrogenase and the redox states of the cytosol and mitochondria could be determined separately by using appropriate redox state couples.

Adult Sprague-Dawley rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and after cannulation of the trachea the respiration was controlled using a rodent respiration pump. The animals breathed room air for 30 min in the control experiments and 10-11% O₂ in nitrogen in the hypoxia experiments. Some of the hypoxia groups had been placed in a hypobaric pressure chamber at 50.5 kPa for 24 h prior to the 30-min period in the respirator. At the end of this controlled ventilation in the respirator one lobe of the liver was freeze-clamped, and the following metabolite ratios were determined enzymatically: lactate/pyruvate, α -glycerophosphate/dioxyacetonephosphate and 3-hydroxybutyrate/acetoacetate.

All the redox pairs measured became more reduced under hypoxic conditions. After the acute 30-min period in the respirator under 10-11% O₂ the lactate/pyruvate ratio increased from the control value of 13.5 to a figure of 80.4, the α -glycerophosphate/dioxy-

uptake previously determined. The heart rate of the subjects was 110–130/min, and the duration of the exercise was 60 min.

The reproducibility of the time activity curves of the total lipolytic activity and of the hepatic and extra hepatic lipases after heparin administration was good both at rest and during exercise. However, rather marked inter individual differences were observed both in the form of the curves and in the peak activity.

The physical exercise used appeared not to exert any clear-cut changes on the enzyme activity curves. However, in obese subjects the peak activities were 40–60% higher than in non-obese subjects and the relative increase was most remarkable in extra-hepatic lipoprotein lipase. Typical enzyme activity curves are shown in Fig. 1.

According to the present results the immediate response to physical exercise of the lipolytic activity released by heparin is small. Studies concerning possible adaptive effects of long-term physical training on enzymes of triglyceride metabolism are now in our research program.

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D 10

Human Skeletal Muscle: Subgrouping of Fast Twitch Fibres

By G. SJØGAARD, M. HOUSTON, E. NYGAARD-JENSEN and B. SALTIN. *August Krogh Institute, University of Copenhagen, Copenhagen, Denmark.*

It is generally accepted that human skeletal muscle is composed of two major fibre types: slow twitch (ST) and fast twitch (FT). An ongoing discussion is, what speaks in favor of 2 subgroups of human FT fibres in accordance with other species.

From histochemical stainings for myofibrillar ATPase (Brooke and Kaiser 1970) FT fibres can with great certainty be classified into two groups. Based on this classification, significant differences of FTa and FTb fibres have been demonstrated with respect to: 1) cross sectional area, 2) recruitment pattern and 3) biochemically analyzed enzyme activities (Essén *et al* 1975).

In the present study it was investigated whether the subgroups of FT fibres can be identified reliably based on histochemical stainings for oxidative and glycolytic enzyme activities. Muscle biopsy samples were obtained from the quadriceps femoris muscle (vastus lateralis) from 4 untrained women, 4 trained men and 4 well trained orienteers. Serial cross sections were histochemically stained for NADH D, α -GP-D and myofibrillar ATPase (after preincubation of respectively pH=10.3, 4.6 and 4.3). FT fibres were identified from the ATPase (pH=10.3 and 4.3) stains and then divided into two groups based on the staining intensity of NADH D, α -GP D and ATPase (pH=4.6). Three persons worked independently each with one of the three stains. The classification of each fibre

D 13

Rapid Determination of Cardiac Output in Small Animals from Dye Dilution MeasurementsBy L. STÅLZ, *Department of Physiology University of Göteborg Sweden*

Everybody who has used the dye dilution method for cardiac output (CO) determination knows that the calculations are quite time consuming, or involve expensive instruments. An inexpensive system, primarily designed for the use in rats, is described which makes it possible to perform frequent CO determinations with calculation of the CO values within 30 minutes after the injection, strictly according to the Stewart Hamilton method. Originally the most time consuming moments in this method are the logarithmic plotting and splitting of the density curve together with the determination of area under curve. However if it is known where the density curve is monoexponential, the area under the curve can be rapidly determined with an integrator and two points on the density curve.

The system for CO measurement consists of a step dispenser injection device, a densitometer with a section arrangement, an electronic unit, a three channel writer and a programmable pocket calculator. The electronic unit provides the three channel writer with signals for 1) the cardiogreen density curve, 2) the integral of the curve and 3) its logarithm. The unit also includes an automatic baseline reset. From the down-slope of the logarithmic curve where it forms a straight line, it can be judged which part of the density curve that is strictly monoexponential. Two points on this monoexponential part of the density curve completely determine the tail function, from which the tail area can be calculated. Now the total area under the curve is determined as the integrator's value where the tail starts plus the tail area. Calculation of flow from a few parameters and curve points involves some arithmetics which are rapidly performed on a programmable pocket calculator.

Two factors must be known prior to experiment, namely the sensitivity of the integrator and that of the densitometer. The sensitivity of the integrator is determined as the ratio between the integrator's deflection and the corresponding density curve area. Calibration of the densitometer can be difficult because of erythrocyte sedimentation if blood becomes trapped in the densitometer chamber. Therefore plasma is used for calibration instead of whole blood, and the densitometer lamp is then dimmed so the same amount of light falls on the photocell as with blood. The results agree closely with the earlier used computer based way of calculating CO.

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D 14

Volumes of Saliva per Shock when Stimulating the Parasympathetic Nerve of the Dog's Submandibular Gland at Different FrequenciesBy P. GÖRSTRUP and N. EMMELIN, *Department of Physiology and Biophysics University of Lund Sweden*

In 2 dogs under chloralose-urethane anaesthesia a submandibular duct was cannulated and connected to a bottle in which saliva displaced distilled water. The chorda-lingual nerve

acetonphosphate ratio from 12.8 to 71.8 and the 3-hydroxybutyrate/acetoacetate ratio from 1.40 to 3.73. After the 24-h hypoxic period at 50.5 kPa followed by 30 min in the respirator under 10–11% O₂, however, the corresponding ratios were markedly lower than those obtained in the animals not previously exposed to hypoxia.

The results demonstrate a significant increase in both mitochondrial and cytosolic free NADH/NAD ratios even in moderate hypoxia, but these redox changes are smaller in partially adapted animals.

D 12

Hepatic Lipogenesis in the Rat under Chronic Severe Hypoxia

By V. L. KINNULA, M. J. SAVOLAINEN and I. HASSINEN *Departments of Physiology and Medical Biochemistry, University of Oulu, Finland*

Severe hypoxia has been observed to cause triglyceride accumulation in rat liver, and one reason for steatosis seems to be a decrease in fatty acid oxidation capacity (Kinnula and Hassinen 1977). Another possible reason for this fat accumulation is increased lipogenesis in the liver.

In this study we measured hepatic lipogenesis *in vivo* by determining the incorporation of ³H into long-chain fatty acids, as described by Lowenstein *et al.* (1975). The animals were kept in a hypobaric pressure chamber at 40.8 kPa for seven days. On the eighth day they were injected intracardially with 5 mCi of ³H₂O. They were then returned to the hypobaric pressure chamber still at 40.8 kPa, and liver and blood samples were obtained 60 min after the injection under ether anesthesia. Two control groups were used: normal-fed and pair-fed groups. The synthesis rates for hepatic fatty acids and non-saponifiable lipids were calculated as the amounts of ³H₂O incorporated in the lipid fractions. The activity of phosphatidate phosphohydrolase (EC 3.1.3.4), which seems to be a rate-limiting step in diacylglycerol and triacylglycerol formation in liver cells (Lamb and Fallon 1973), was also determined from the supernatant fraction after a seven-day hypoxic period at 40.8 kPa (Hosaka *et al.* 1975; Savolainen 1977).

It was observed that lipogenesis does not increase after a hypoxic period of seven days; the synthesis of hepatic fatty acids being 3.37 ± 1.70 μ mol of ³H₂O incorporated per h per g wet wt. of liver in the control group, 2.31 ± 0.97 μ mol in the pair-fed control group and 2.63 ± 0.33 μ mol in the hypoxic group. None of these differences were significant. Phosphatidate phosphohydrolase activity had not increased after seven days either; the activity being 97.9 ± 21.1 nmol P per min per g wet wt. of liver in the control group and 79.3 ± 25.7 nmol P in the hypoxic group, the difference being non-significant.

These preliminary results indicate that chronic oxygen deficiency has no effect on hepatic lipogenesis *in vivo*.

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D 15

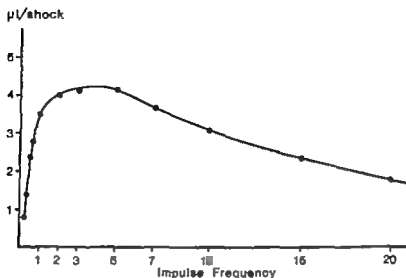
The Effect of Trauma on the Microcirculation in Skeletal Muscle

By DAVID H. LEWIS. *Clinical Research Center University Hospital Linköping
Sweden*

In the presence of trauma or hemorrhage the microcirculation of normal skeletal muscle responds initially with at least three compensatory reactions mediated centrally via the sympathetic vasoconstrictor nerves. These include an increase in resistance to flow, decrease in capacitance and an increase in the ratio of precapillary to postcapillary resistance. These responses are compensatory in that they help to maintain blood pressure and blood volume centrally and move extravascular fluid into the intravascular space. When skeletal muscle is itself traumatized the microcirculation of the injured muscle demonstrates an immediate, marked, transient vasodilatation which responds poorly or not at all to sympathetic vasoconstrictor stimuli and sympathomimetic agents. In anesthetized dogs (or pigs) soft tissue trauma to the hind leg causes a biphasic vasodilatory response: an initial vasodilatory spike which after 5-15 min goes over to a more moderate vasodilation lasting 60-90 min. The vasodilatation is not simply a release from sympathetic vasoconstrictor tone since trauma after acute post-ganglionic sympathectomy causes an identical vasodilatation. Administration of large doses of aprotinin (Trasyol®) a broad spectrum protease inhibitor before trauma reduces markedly or abolishes the "spike" response without altering the second phase. Aprotinin given after trauma has no effect. Inhibitors of serotonin, histamine (H_1 -receptor) and prostaglandins given before trauma do not alter the vasodilatation. During the initial vasodilatory spike venous blood draining the injured area, injected close intraarterially into normal skeletal muscle, causes vasodilatation. This response can be heightened and prolonged by prior administration of dimeric captopril (British antilewisite) to the recipient. Dimeric captopril is known to inhibit the breakdown of bradykinin by inactivating carboxypeptidase. The response is not affected by giving inhibitors of serotonin, H_1 -histamine receptors, or prostaglandins. The data taken together suggest that the vasodilatation seen with trauma is due to activation of the kallikrein-kinin system with release of bradykinin. Fracture of bone without accompanying soft tissue damage does not cause vasodilatation. In addition to microvascular changes there is a prolonged dilatation of the arterial tree in the injured limb extending proximal to the site of injury (ascending vasodilatation). The response to gunshot wounds is similar to that seen with soft tissue trauma. Further elucidation of the etiology and significance of the vasodilator response to trauma is of interest, since it appears not to be related to the tissue's metabolic needs and may have a negative effect on the body's homeostatic mechanisms.

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was stimulated at frequencies between 0.2 and 20 Hz. Drops of water of known size falling from an outlet were recorded and the secretory rate was estimated when it had settled down to a steady level initially it was often high an effect attributed to expulsion of saliva by myoepithelial contraction (Emmelin Gjörrstrup and Thesleff 1977) For each stimulation frequency the volume secreted was calculated as μl per shock The Figure shows that the largest yields per shock were obtained at stimulation frequencies between 1 and 8 Hz This is interesting in view of the fact that previous experiments on this gland indicate that maximal secretory responses to feeding are brought about by secretory impulses of frequencies which do not exceed 8 Hz at 1 Hz about 10 percent of the maximal secretory flow is obtained (Emmelin and Holmberg 1967) It is obvious that the parasympathetic acinar secretory nerves have their largest effect expressed as volume secreted per impulse within a range where they operate physiologically to evoke a fairly slow moderate or rapid salivary flow At frequencies below 1 Hz the curve falls steeply It is possible however that here sympathetic impulses contribute causing an "augmented secretion" (Emmelin and Gjörrstrup 1976)

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D 15

The Effect of Trauma on the Microcirculation in Skeletal Muscle

BY DAVID H. LEWIN. *Clinical Research Center University Hospital Linköping*
S edm

In the presence of trauma or hemorrhage the microcirculation of normal skeletal muscle responds initially with at least three compensatory reactions mediated centrally via the sympathetic vasoconstrictor nerves. These include an increase in resistance to flow, decrease in capacitance and an increase in the ratio of precapillary to postcapillary resistance. These responses are compensatory in that they help to maintain blood pressure, move blood volume centrally and move extravascular fluid into the intravascular space. When skeletal muscle is itself traumatized the microcirculation of the injured muscle demonstrates an immediate marked transient vasodilatation which responds poorly or not at all to sympathetic vasoconstrictor stimuli and sympathomimetic agents. In anesthetized dogs (or pigs) soft tissue trauma to the hind leg causes a biphasic vasodilatory response, an initial vasodilatory "spike" which after 5-15 min goes over to a more moderate vasodilatation lasting 60-90 min. The vasodilatation is not simply a release from sympathetic vasoconstrictor tone since trauma after acute post-ganglionic sympathectomy causes an additional vasodilatation. Administration of large doses of aprotinin (Trasylo[®]) a broad spectrum protease inhibitor before trauma reduces markedly or abolishes the "spike" response without altering the second phase. Aprotinin given after trauma has no effect. Inhibitors of serotonin, histamine (H_1 -receptor) and prostaglandins given before trauma do not alter the vasodilatation. During the initial vasodilatory spike, encasement draining the injured area, injected close intraarterially into normal skeletal muscle causes vasodilatation. This response can be heightened and prolonged by prior administration of dimeric caprol (British antilewisite) to the recipient. Dimeric caprol is known to inhibit the breakdown of bradykinin by inactivating carboxypeptidase. The response is not affected by giving inhibitors of serotonin, H_1 -histamine receptors or prostaglandins. The data taken together suggest that the vasodilatation seen with trauma is due to activation of the kallikrein-kinin system with release of bradykinin. Fracture of bone without accompanying soft tissue damage does not cause vasodilatation. In addition to microvascular changes there is a prolonged dilatation of the arterial tree in the injured limb extending proximal to the site of injury (ascending vasodilatation). The response to gunshot wounds is similar to that seen with soft tissue trauma. Further elucidation of the etiology and significance of the vasodilator response to trauma is of interest, since it appears not to be related to the tissues' metabolic needs and may have a negative effect on the body's homeostatic mechanisms.

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An Implantable Osmometer for Determination of Interstitial Fluid Colloid Osmotic Pressure

By R. K. REED and K. AUKLAND *Institute of Physiology University of Bergen
Årstadveien 19 5000 Bergen Norway*

The colloid osmotic pressure of interstitial fluid (COP) remains an uncertain factor in the Starling equilibrium across the capillary walls. To obtain a direct estimate of COP, we have constructed an implantable colloid osmometer making use of Amicon UM10 hollow fibers. As shown in Fig. 1 a 0.2 mm steel cannula provided with a sidehole is inserted into a 6–8 mm long fiber. The fiber ends are glued to the cannula, which is connected to a pressure transducer through a PE 20 catheter. The system is filled with saline before use.

The instrument was tested against a membrane colloid osmometer (Aukland and Johnsen 1974) on III sera from humans and rats giving a regression coefficient of 1.04 and a correlation coefficient of 0.99.

Acute implantation through a 1.6 mm (o.d.) thick cannula in rats gave average pressures of -10.8 (SD 1.4 $n=18$) in subcutis and -8.6 mm Hg (SD 2.1 $n=17$) in skeletal muscle. The response time was 10–15 min both *in vivo* and *in vitro*. Hydrostatic tissue fluid pressures obtained by implanting the osmometer after perforating the membrane averaged -0.65 and -0.50 mm Hg in subcutis and muscle respectively. Subtracting these values gives COP_i of about 10 and 8 mm Hg in the two tissues. These values are comparable to the average of 10.2 mm Hg obtained by Stromberg and Wiederhielm (1976) with chronically implanted colloid osmometers in rabbit subcutis and they agree well with COP of fluid collected by wick-technique from subcutis and muscle in rats (Johnsen 1974).

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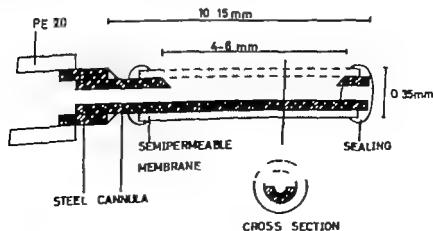


Fig. 1 Longitudinal and cross section through implantable colloid osmometer

D 17

Influence of Detergents on Mg^{2+} + Ca^{2+} -activated ATPase from Bovine Brain Microsomes

By T. SÆRMARK and H. VILHARDT *Institute of Medical Physiology C University of Copenhagen, Denmark*

In the presence of ouabain and mitochondrial ATPase inhibitors bovine brain microsomes display membrane bound ATPase activity dependent on Ca^{2+} and Mg^{2+} . It is shown that this activity can be solubilized by ionic and non-ionic detergents. Triton X 100 treatment resulting in the most stable soluble form of the enzyme.

Bovine brain microsomes were treated with deoxycholate (DOC) at various concentrations at 0°C for 20 min. at pH 8.2 and then centrifuged at $100\,000\times G$ for 60 min. at 4°C. Small concentrations (0.15%) solubilized 60% of the membrane proteins but only 10% of the ATPase activity despite some activation of the enzyme. Higher concentrations were able to solubilize more of the enzyme but also inhibited the ATPase activity. At a concentration of 1% DOC 30% of the original activity could be recovered in the supernatant. The DOC-solubilized enzyme was unstable with time.

The microsomes were incubated with various concentrations of Triton X 100 at 20°C for 30 min. followed by centrifugation. Small concentrations of Triton X 100 did not activate the enzyme. At higher concentrations (0.6%) 55% of the protein of the preparation was solubilized. At the same time 30% of the initial ATPase activity could be recovered in the supernatant. The solubilized preparation was relatively stable with time. After 72 h the enzyme activity decreased to 50% of the initial activity when stored at 4°C and to 90% when stored at -22°C. On longer storage the preparation had a tendency to become cloudy however without substantial loss of activity. Addition of phospholipids to the preparation partially restored enzyme activity. Ca^{2+} (1 mM) decreased the stability of the Triton X 100 treated enzyme. Sonication of the microsomes in the presence of detergents was without effect on solubilization.

Polyacrylamide gel electrophoresis of the Triton X 100 solubilized microsomal fraction revealed 8 bands with the ATPase being the most prominent. Analytical isoelectric focusing (pH 3-10) showed 15 bands all below pH 7.5. When the preparation was subjected to preparative isoelectric focusing in granulated gel beds the ATPase activity was recovered at pH 4.8 and 6.4.

The present results show that by selecting suitable detergents and concentrations it is possible to solubilize membrane bound enzymes. This procedure opens up the possibility for further purification of the enzyme.

D 18

Rapid Restoration of Choline Acetyltransferase Activity in the Partially Denervated Urinary Bladder

By HEATHER BAKER, J. EKSTRÖM and S. P. MANN *From ARC Institute of Animal Physiology Babraham Cambridge England and Department of Physiology and Biophysics University of Lund Sweden*

An Implantable Osmometer for Determination of Interstitial Fluid Colloid Osmotic Pressure

By R. K. REED and K. AUKLAND *Institute of Physiology University of Bergen, Årstadveien 19 5000 Bergen Norway*

The colloid osmotic pressure of interstitial fluid (COP) remains an uncertain factor in the Starling equilibrium across the capillary walls. To obtain a direct estimate of COP, we have constructed an implantable colloid osmometer making use of Amicon UM10 hollow fibers. As shown in Fig. 1 a 0.2 mm steel cannula provided with a sidehole is inserted into a 6–8 mm long fiber. The fiber ends are glued to the cannula, which is connected to a pressure transducer through a PE 20 catheter. The system is filled with saline before use.

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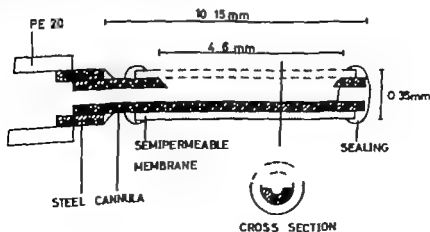


Fig. 1 Longitudinal and cross section through implantable colloid osmometer

were taken through a mask fitted around the dog's nose. The dog breathed alternating 21% 100% 21% 100% O_2 for each 6 min periods. Measurements were recorded on a vibrometer every 2nd min for coronary blood flow (CBF) and aortic pressure (AP). From these curves mean diastolic CBF and mean diastolic AP were computed by planimetry. CBF during 1% was 31.7 ± 5.7 (SE) ml/min and decreased significantly ($p < 0.05$) to 27.8 ± 4.5 (SE) ml/min. Conductance (CBF/AP) decreased ($p < 0.05$) from 460 ± 078 to 334 ± 073 ml/min/mmHg during 21% and 100% O_2 respectively. Oxygen delivery (CBF arterial O_2 content) remained unaffected. It is concluded that in the normal dog least O_2 acts as a vasoconstrictor autoregulating CBF according to O_2 needs.

D 20

Leg Citrate Metabolism in Relation to Diet in Man

By E. JANSSON and L. KAUSER, *Karolinska Institute Departments of Clinical Physiology at Karolinska Hospital and Serafimer Hospital Stockholm Sweden*

Citrate is considered as an important intermediary in the regulation of cell metabolism. If plasma free fatty acids (FFA) are increased citrate accumulates in heart and red skeletal muscle in the rat (Rennie et al. 1976). From this and in vitro experiments it is proposed that increased intracellular citrate concentration inhibits glycolysis at the phosphofructokinase step and interconversion of the inactive to the active form of pyruvate dehydrogenase (Garland et al. 1963 and Wayne et al. 1973).

The aim of present study was to relate leg citrate metabolism to type of diet. 10 healthy subjects were studied after 5 days on fat or carbohydrate rich diet. Respiratory exchange ratio, arterial-femoral venous differences of glucose, lactate, citrate and pyruvate and arterial concentrations of FFA were measured at rest and during exercise.

The respiratory exchange ratio was lower after fat diet at rest and during exercise. Arterial concentration of FFA and citrate were higher after fat and lactate and pyruvate higher after carbohydrate diet both at rest and during exercise. The release of citrate

Table 1 Arterial concentrations and arterial-venous differences of blood citrate and pyruvate at rest and during exercise after fat or carbohydrate diet

	Blood citrate, Fat diet	$\mu\text{mol/l}$ Carb. diet	Blood pyruvate, Fat diet	$\mu\text{mol/l}$ Carb. diet
artery rest	58.3 ± 13.1	$51.4 \pm 13.3^*$	31.9 ± 7.7	$70.4 \pm 34^{**}$
artery exercise 5 min	58.8 ± 12.8	53.5 ± 13.6	136 ± 19	$164 \pm 37^{**}$
artery exercise 23 min	74.1 ± 18.9	$66.4 \pm 17.6^{**}$	143 ± 28	$181 \pm 43^{**}$
ve rest	-14.3 ± 5.8	-12.5 ± 4.1	$+4.3 \pm 4.8$	8.4 ± 12
ve exercise 5 min	-3.2 ± 1.3	$-7.6 \pm 1.0^*$	-16.7 ± 8.3	$-4.3 \pm 10^*$
ve exercise 23 min	-3.2 ± 1.8	-4.0 ± 2.2	-5.8 ± 12	-4.8 ± 11

Values are mean \pm SD. Levels of significant difference between fat and carbohydrate rich diet are: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

Recently the cholinergic innervation of the urinary bladder of the rat was studied by determining the activity of the acetylcholine-synthesizing enzyme choline acetyltransferase, after various surgical procedures (Ekström and Elmér 1977). In this connection the observation was made that the choline acetyltransferase activity was decreased somewhat in bladders examined 3 days after cutting the hypogastric nerve bilaterally but not in those examined 8 days after the operation. At the later stage the enzyme activity tended, in fact, to be higher in the operated bladders than in the controls. The initial fall in enzyme activity was taken as evidence for the presence of cholinergic nerve fibres for the bladder in the hypogastric nerve. The fact that no decrease in enzyme activity was in hand at 8 days may be explained by an increased nervous activity in remaining nervous pathways induced by the loss of the hypogastric innervation of the bladder (see Ekström 1975). The main route for the cholinergic nerves of the bladder is via the pelvic ganglion as indicated by the very profound fall in enzyme activity after bilateral extirpation of this structure. The choline acetyltransferase activity has now been investigated at various times after unilateral removal of the pelvic ganglion to see whether also under this condition a restitution of enzyme activity from a low level could be provoked.

The enzyme activity estimated by a radioactive method was examined in bladders of adult male rats 3, 6 and 25 days postoperatively. At each observation the enzyme activity of 8 operated animals was compared with that of 8 control litter mates and expressed as a percentage. The mean enzyme activity was (operated in % of control) at 3 days 58 ± 4 at 6 days 75 ± 4 and at 25 days 86 ± 8 . The percentage figures obtained at 6 and 25 days differ significantly from the figure at 3 days, the p-levels being <0.02 and <0.01 respectively. Thus the results show that in a bladder deprived of one of the main routes for cholinergic nerves the enzyme activity increases rapidly from a low level. This increase may be restricted to the nerves present, it may also reflect collateral sprouting. Both these possibilities may be attributed to enhanced nervous activity in the remaining pathway.

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D 19

Oxygen a Coronary Vasoconstrictor

By FLEMMING BONDE PETERSEN and H. JAMES BARNARD. August Krogh Institute University of Copenhagen, Denmark, and Division of Thoracic Surgery, University of California Medical Center, Los Angeles, USA.

Seven mongrel dogs had a flow probe placed around the left coronary artery and a pressure transducer placed in the wall of the aortic arch. Two weeks after the operation, resting values were measured during breathing of either 21% or 100% O₂, administered from pres-

through a mask fitted around the dog's nose. The dog breathed alternating 100% & 21% O₂ for each 6 min periods. Measurements were recorded on a every 2nd min for coronary blood flow (CBF) and aortic pressure (AP). From venous diastolic CBF and mean diastolic AP were computed by planimetry. At 21% was 31.7 ± 5.7 (SE) ml/min and decreased significantly ($p < 0.05$) to 17.1 (SE) ml/min. Conductance (CBF/AP) decreased ($p < 0.05$) from 460 ± 078 ml/min/mmHg during 21% and 100% O₂ respectively. Oxygen delivery (aortic O₂ content) remained unaffected. It is concluded that in the normal dog acts as vasoconstrictor autoregulating CBF according to O₂ needs.

D 20

Cell Metabolism in Relation to Diet in Man

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Citrate is considered as an important intermediary in the regulation of cell metabolism. When free fatty acids (FFA) are increased citrate accumulates in heart and red skeletal muscle in the rat (Rennie et al. 1976). From this and *in vitro* experiments it is proposed that increased intracellular citrate concentration inhibits glycolysis at the phosphofructokinase and interconversion of the inactive to the active form of pyruvate dehydrogenase (Garland et al. 1963 and Wayne et al. 1973).

The aim of present study was to relate leg citrate metabolism to type of diet. 10 healthy subjects were studied after 5 days on fat or carbohydrate rich diet. Respiratory exchange ratio, femoral-venous differences of glucose, lactate, citrate and pyruvate and arterial concentrations of FFA were measured at rest and during exercise. Respiratory exchange ratio was lower after fat diet at rest and during exercise. Arterial concentration of FFA and citrate were higher after fat and lactate and pyruvate after carbohydrate diet both at rest and during exercise. The release of citrate

Arterial concentrations and arterial-venous differences of blood citrate and pyruvate at rest and during exercise after fat or carbohydrate diet

	Blood citrate, Fat diet	$\mu\text{mol/l}$ Carb. diet	Blood pyruvate, Fat diet	$\mu\text{mol/l}$ Carb. diet
at rest	38.3 ± 13.1	51.4 ± 13.3	31.9 ± 7.7	70.4 ± 24.0**
exercise 5 min	58.8 ± 12.8	53.3 ± 13.6	136 ± 19	164 ± 27*
exercise 23 min	74.1 ± 18.0	66.4 ± 17.6**	143 ± 28	181 ± 43
at rest	-14.3 ± 5.0	-12.3 ± 4.1	+4.3 ± 4.8	+8.4 ± 12
exercise 5 min	-3.2 ± 1.5	-1.6 ± 1.0*	-16.7 ± 8.3	-6.5 ± 10*
exercise 23 min	-3.2 ± 1.8	-4.0 ± 2.2	-5.8 ± 12	-4.8 ± 11

are mean ± SD. Levels of significant difference between fat and carbohydrate rich diet are: (*), $p < 0.01$ (**) and $p < 0.001$ (***)

pyruvate and lactate at rest, were not different between diets. After 23 min of exercise there were no differences in citrate and pyruvate release but lactate release was much higher after carbohydrate. However after 5 min of exercise there was greater release of citrate and pyruvate and lower release of lactate after fat than carbohydrate diet (Table 1). This might indicate that high levels of FFA at onset of exercise leads to increased synthesis of citrate increasing the level of acetyl-CoA. Increased pyruvate release in spite of low lactate release indicates decreased pyruvate oxidation rate.

In conclusion the results are consistent with the theory that increased oxidation of fatty acids elevate citrate concentration thereby inhibiting glycolysis and pyruvate oxidation.

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D 21

A Study of Thalamo-cortical Recruiting Responses during the Reserpine State in the Cat

By A. LAIHINEN and P. VALLEALA. *Department of Physiology, University of Turku, Finland*

In this study the state induced with reserpine—reserpine state—is considered an entity of its own as contrasted against wakefulness, slow wave sleep (S) and paradoxical sleep (PS). Reserpine in a dose of 0.50 to 0.75 mg/kg induces in the cat a longlasting sedation and ponto-geniculo-occipital wave activity (PGO) which normally occurs during PS and the final phase of S immediately preceding PS. In this study the "early" reserpine state is characterized by electrocortical desynchronization, PGO activity with stereotype rhythm and moderate neck muscle tone and the "late" reserpine state by temporary synchronization of electrocortical activity and the first signs of PS. S and PS do not occur during the early reserpine state whereas the late reserpine state means the gradual restitution of normal sleep.

Thalamo-cortical recruiting responses (RRs) most typically encountered in barbiturate anesthesia and during S can be elicited in a modified form during PS. Recently it was shown that RRs do not usually occur simultaneously either with PGO waves during PS (PGO_{PS}) or with PGO waves in the early reserpine state (PGO_{RES}) (Laihinen and Valleala 1977). In the present study the relationship of RRs and PGO_{RES} was further analyzed in the late reserpine state.

Serpasil® was given 0.75 mg/kg/i.p. PGO_{RES} waves were recorded in the geniculate nuclei. The centre median nucleus was stimulated at a rate of 7-9 Hz. The RRs were recorded from the anterior sigmoid gyrus. During the late reserpine state when the electrocortical activity was synchronized there were plenty of PGO_{RES} waves not only in the presence of spontaneous spindles but also during RRs i.e. the mutual exclusion found in

the early reserpine state did not exist. When PS reappeared that exclusion could be observed.

Normally spontaneous spindle and PGO_{res} activity can occur simultaneously during the transition from S to PS. This is analogical to the coincidence of spindle-like activity (spontaneous spindles and RRs) and PGO_{res} waves during the late reserpine state. In both the situations neither PS nor S has an absolute dominance. It is a dynamic equilibrium in certain components of both S and PS-spindle activity and PGO waves even simultaneously and could be related to the changing concentrations of the relevant transmitters.

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D 22

Morphology and Function of M. Biceps Brachii in Man. A Worth-while Experimental Model?

By E. NYGAARD, K. JØRGENSEN, M. HOUSTON, J. SUZUKI and B. SALTIN *August Krogh Institute University of Copenhagen, Copenhagen, Denmark*

Skeletal muscle of most species comprise fibres with different characteristics. A subdivision in one slow (ST) and two fast twitching (FT) fibres is usually satisfactory. A close coupling between histochemical and biochemical characteristics of the various fibre types and their function has been established in muscles from rat, guinea pig and cat (for ref. see Burke and Edgerton, 1975).

In skeletal muscle of man three fibre types have also been described, although the differences between the two fast twitching fibres (FTa and FTb) has not yet been established (for ref. see Saltin *et al.* 1977). Indirect evidence for a coupling between muscle fibre characteristics and function of skeletal muscle of man are available from studies of glycogen depletion patterns, and of single motor unit recordings (Grimsby and Hannertz 1977). Further, Thorstensson (1976) using an isokinetic device and knee extension has found certain relationships between fibre composition of the vastus lateralis and certain capacities of the thigh muscle. However, there are some limitations to this model, one being that different muscles are involved in the knee extension and another that less than half the maximal velocity can be achieved.

To overcome some of these difficulties we have chosen to use a modification of Hill's model to establish the force-velocity relationship for arm flexion in six adult healthy subjects. Muscle biopsies were obtained from the central part of the biceps brachii muscle for histochemical and biochemical analysis. Estimation was made of the cross-sectional area of the muscle mass. To evaluate endurance capacity dynamic work was performed in extension at 20% MVC.

Results: Fibre distribution averaged 45% ST fibres (range 14-63%), 27% FTa (14-34%) and 28% FTb fibres (13-57%). Fibre distribution did not correlate with any functional parameters. Mean fibre area correlated with cross section of the muscle mass as well as

with MVC ($p < 0.01$) the correlation being most pronounced between mean area of F fibres and MVC ($r = 0.88$ $p < 0.001$). Total FT area of the muscle correlated with strength at 50% of maximal velocity as well as with velocity at 50% MVC ($p < 0.01$) the FTa fibre showing a higher correlation than the FTb fibres. No relationship was found between the oxidative characteristics (proportion of ST fibres, relative ST area, capillarization and SDH activity) and endurance capacity of the muscle (performance time, total work, rise in oxygen uptake and heart rate).

In conclusion it can be stated that significant relationships have been found between some fibre characteristics of the biceps brachii and its function, indicating that the structure of this muscle during arm flexion may well serve as a good experimental model. The lack of relationship between certain variables is most likely due to a relatively large variability in the measurement of some of these parameters in comparison to the rather narrow range for these variables found in the few subjects studied.

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D 23

Simultaneous Measurements of Capillary Diffusion and Filtration Exchange at Alterations in the Capillary Permeability-Surface Area Product (PS) and During Shifts in Filtration-Absorption

By B. RIPPE, A. KAMIYA and B. FOLKOW. *Department of Physiology, University of Göteborg, Sweden*

Only seldom has it been possible to compare quantitatively and simultaneously capillary diffusion and filtration events in well defined vascular beds during various situations. Therefore the diffusion exchange of Cr-EDTA, measured with a colorimetric variant of the single injection indicator diffusion method (Rippe and Stage 1976) was followed simultaneously with estimations of the capillary filtration capacity (CFC) using the "isogravimetric" rat hindquarter preparation during artificial perfusion and maximal dilatation. Measurements were performed at constant flow and during 1) alterations of permeability (i.e. infusion of histamine), 2) alterations of perfused capillary wall area (graded rarefaction of the capillary network by microspheres $\varnothing \sim 15 \mu\text{m}$) and 3) shifts in filtration-absorption.

At maximal vasodilatation during "control" CFC was $0.037 \pm 0.001 \text{ ml/min} \times \text{mm Hg} \times 100 \text{ g}$ and PS for Cr-EDTA was $5.67 \pm 0.13 \text{ ml/min} \times 100 \text{ g}$. Histamine increased CFC nearly fourfold while PS for Cr-EDTA remained almost unchanged. In contrast alterations in capillary wall area, as reflected by roughly reciprocal changes in flow resistance

from control, resulted in proportional changes in PS for Cr EDTA while the CFC changes are always relatively smaller. Transcapillary solute transfer from vessels to tissue was only little affected by shifts in capillary filtration-absorption. During filtration PS for Cr EDTA increased only slightly reaching 6-7% increase at a filtration rate of $1 \text{ ml/min} \times 100 \text{ g}$. During absorption the capillary to tissue transfer of Cr EDTA remained essentially unchanged.

The present findings may suggest that 1) histamine increases capillary permeability by increasing the number and/or mean radius of the large pores which because of their relative size are of little importance for small molecular diffusion exchange but, by virtue of their large radius, are highly important for convective and macromolecular exchange. Alterations of the perfused capillary wall area are closely reflected by changes in PS but, somewhat underestimated by CFC measurements since CFC estimations are "flow-limited". 3) Blood to tissue diffusion of small molecules is only little modified by convection effects, but this is not necessarily true for back diffusion.

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D 24

Characteristics of Cardiac Vagal Afferents in Normotensive and Hypertensive Rat

By P. THORÉN, S. E. RICKSTEN and E. NORRÉSON, Department of Physiology, University of Göteborg, Göteborg, Sweden

Cardiac receptors with vagal afferents were first described by Paintal and has been studied mainly in dogs and cats (Paintal 1973). The heart of cats and dogs also has a large population of atrial (Thorén 1976) and ventricular receptors (Thorén 1977) with non-medullated axons.

The aim of the present study was to examine the characteristics of the vagal cardiac receptors in normotensive and hypertensive rats. The rats were anaesthetized with nembutal at a dose of 50 mg/kg b.w. intraperitoneally. Thorax was opened and a catheter was inserted in the left atrium via a lung vein. The aortic pressure was measured via a femoral artery. Snakes were placed round the ascending aorta and the pulmonary artery. Filaments were dissected from the right vagus and tested for activity coming from the heart. Dissections were continued until adequate single fiber recordings were obtained.

Results in normotensive rats: Recordings were obtained from 13 receptors in 9 rats. 11 receptors were located to the left atrium and 2 receptors to the right atrium. No receptors were found in the ventricles. The conduction velocities were $0.6-1.3 \text{ m/sec.}$ No receptors with medul-

lated afferents were found. Threshold for activation of the left atrial receptors were 2.5–11.5 mm Hg in mean left atrial pressure and maximal frequencies varied between 8 and 55 Hz. 10 of 13 receptors showed a clear cardiac rhythmicity upon activation.

Hypertensive rats. Recordings were obtained from 4 left atrial receptors in 4 spontaneous hypertensive rats. These receptors have thresholds of 9–12 mm Hg.

Conclusion. The cardiac vagal receptors in the rat seem to be located mainly in the atria and are connected to afferent non-medullated afferent nerve fibers. Preliminary experiments indicate a higher threshold of these receptors in the hypertensive rat.

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D 25

Sodium Balance During Development of Hypertension in Spontaneously Hypertensive Rats (SHR)

By S. LUNDIN, H. HERLITZ, G. BERGLUND, S. E. RICKSTEN, B. GÖTHBERG and M. HALLBÄCK, *Departments of Physiology and Med. Clin. I, University of Göteborg, Göteborg, Sweden.*

The mechanisms leading to primary hypertension in man and in SHR are still poorly understood. SHR display signs of enhanced sympathetic activity and plasma volume is slightly lowered (Rippe, Lundin and Folkow 1977) but in the Milan hypertensive strain of rats (MHS) the pressure rise is associated with sodium retention and plasma volume increase (Blanchi *et al.* 1975). With increasing severity of hypertension, man displays a decreasing ability to excrete salt and water and an increasing renal resistance (Berglund *et al.* 1977). Thus, both MHS and man display signs of an altered renal handling of sodium, initially or along the course of hypertension, and the question arises whether also SHR show similar alterations of either primary or secondary nature.

A comparison was therefore performed concerning sodium handling in SHR and normotensive control rats (NCR) primary in early phases of SHR hypertension. The rats were kept in metabolic cages and sodium intake, urinary and faecal sodium excretion, plasma renin activity and urinary aldosterone excretion were determined at the age of 6 weeks, 3 and 7 months. Constant flow perfusions of the kidneys were also performed to determine total renal vascular resistance in the different age groups.

At six weeks of age, fractional urinary excretion (urinary excretion/intake $\times 100$) in SHR ($29 \pm 2\%$) was lower ($p < 0.001$) than in NCR ($57 \pm 1\%$) while fractional faecal excretion was higher ($p < 0.001$) in SHR ($27 \pm 2\%$) than in NCR ($12 \pm 1\%$). Total fractional excretion

urinary + faecal excretion/intake $\times 100$) was significantly lower in SHR than in NCR ($56 \pm 2\%$ vs $68 \pm 1\%$) but a true difference in sodium retention ($\mu\text{mol Na/g body weight increase}$) could not be demonstrated due to a correspondingly lower Na intake in SHR. Urine aldosterone activity was slightly higher in SHR (15 per cent). The same excretion pattern was found at 3 months of age although total fractional excretion no longer differed significantly and at 7 months also urinary excretion was largely equal in SHR and NCR. The renal vascular bed showed to be progressively more structurally rebuilt with age.

The results do not suggest any initial sodium retention in SHR, in agreement with reduced plasma volume but the routes of sodium elimination are shifted from kidneys towards gastro-intestinal tract. This can hardly be explained by the observed slight changes in aldosterone but neurogenic hormonal and structural alterations of renal resistance and in pre/postglomerular resistance ratio (Folkow *et al.* 1977) might be involved.

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 RICE, B. S., LUNDQVIST and B. FOLKOW

D 26

Initial Blood Volume and Transcapillary Escape Rate (TER) of Albumin in Young Spontaneously Hypertensive Rats (SHR) Compared with Controls (NCR)

by B. RICE, S. LUNDQVIST and B. FOLKOW *Department of Physiology University of Göteborg Sweden*

As recently been discussed whether a "primary" blood volume increase may be a cause of hypertension, by enhancing venous return and cardiac output which, in turn, could initiate a sustained resistance increase by "whole body autoregulation" (e.g. Guyton *et al.* 1971). Such a chain of events may indeed, be an important initiating element in the spontaneously hypertensive rat (MHS) (Blanchi *et al.* 1975). In SHR, on the other hand, central neurohormonal influences appear to constitute a major initiating element. The question arises whether an early volume increase might contribute also to the development of hypertension.

Plasma and blood volumes were therefore compared in groups of young SHR and NCR, particularly during the phase of rapid pressure rise in SHR (3, 4, 5, 6, 8, 10 weeks of age and 17 months of age). Also TER was measured in 6-10 week old SHR and NCR, since it is commonly increased in man's primary hypertension (Ulrych 1973). After i.v. in-

lated afferents were found. Threshold for activation of the left atrial receptors were 2.5–11.5 mm Hg in mean left atrial pressure and maximal frequencies varied between 8 and 55 Hz. 10 of 13 receptors showed a clear cardiac rhythmicity upon activation.

Hypertensive rats Recordings were obtained from 4 left atrial receptors in 4 spontaneous hypertensive rats. These receptors have thresholds of 9–12 mm Hg.

Conclusion The cardiac vagal receptors in the rat seem to be located mainly in the atria and are connected to afferent non-medullated afferent nerve fibers. Preliminary experiments indicate a higher threshold of these receptors in the hypertensive rat.

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D 25

Sodium Balance During Development of Hypertension in Spontaneously Hypertensive Rats (SHR)

By S. LUNDIN, H. HERLITZ, G. BERGLUND, S. E. RICKSTEN, G. GÖTHBERG and M. HALLBÄCK. *Departments of Physiology and Med. Clin. I, University of Göteborg, Göteborg, Sweden.*

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spatial frequency and the size of the gratings were scaled by the inverse of the cortical magnification factor estimated from the points which Cowey and Rolfs calculated from the data of Brinkley and Lewin (1968) who mapped the phosphene caused in the lower nasal visual field by electrical stimulation of human visual cortex. This way the cortical representations of the gratings were presumably made similar at various eccentricities. For a series of eccentricities of 0, 1.5, 4, 7.5, 14 and 30 deg the following values of magnification factor were assessed: 7.75, 5.25, 3.44, 2.31, 1.24 and 0.49 mm/deg. The scaling of gratings to the inverses of these values was accomplished by keeping the gratings constant and moving the subject closer to the display. The CSFs recorded with scaled cortical representations were almost identical.

Our result implies that a picture can be made equally visible at any eccentricity by scaling its size by the magnification factor for the CSF represents the spatial modulation transfer function of the visual system for near-threshold contrasts. The constancy of the steep CSFs of different but equally large regions of visual cortex indicates a striking cortical homogeneity of image processing machinery.

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D 28

Individual Blood Lactate Response during Exercise and Its Relation to Muscle Fibre Composition

By P. K. PEDERSEN, *Inst. of Physical Education, Odense University, Denmark*

The general pattern of the blood lactate concentration (HLA) during graded exercise is an elevated or slightly increased level during light and moderate exercise followed by a steep increase during severe exercise. Although this is the common pattern, considerable individual differences exist between HLA values at higher relative work loads (percentages of $\dot{V}O_{2\max}$). Studies on muscle fibre composition on humans and animals have shown that muscles are composed by fibres that differ in mechanical as well as biochemical (oxidative-glycolytic) aspects. In order to find out whether variations in muscle fibre composition could account for the individual differences in lactate response to exercise, 17 trained subjects had biopsies taken from the rectus femoris muscle. Based on histochemical staining the muscle fibres were classified in types I, IIA and IIB. Type I is considered to be high oxidative-low glycolytic, type IIA oxidative-glycolytic and type IIB low oxidative-high glycolytic (Essén *et al.* 1975).

The blood lactate response was studied in a progressive bicycle exercise test comprising 1-5 submaximal work intensities followed by a maximal test. The measurements included oxygen uptake as well. From these data individual relative load-blood lactate curves were

jections of I^{125} labelled human serum albumin blood samples were withdrawn 20 mm later for determination of hematocrit and with estimation of blood and plasma volumes from the extent of tracer dilution. TER was estimated from the gradual reduction with time of the tracer concentration in plasma.

The results show that during early age plasma volume was slightly but significantly ($p < 0.001$) lower in all the SHR groups when compared with NCR of identical body weights. Though hematocrit was slightly higher in SHR than in NCR, also blood volume tended to be lower in SHR ($p = 0.06$). First at the age of 7 months when SHR hypertension is fairly advanced with beginning complications, blood and plasma volumes tended to be higher in SHR than in NCR. TER was increased in SHR 19.7 ± 0.8 per cent/hour compared with 16.4 ± 0.7 in NCR ($p < 0.01$).

These results suggest that SHR hypertension unlike that in MHS is not dependent of any "primary" increase in cardiovascular filling. The reduced blood volume, hemoconcentration and increased TER is in agreement with the view that an early enhancement of sympathetic activity is a dominant initiating element in SHR hypertension as this will somewhat raise also venous and capillary pressures in SHR.

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D 27

Cortical Representation of Visual Field predicts the Photopic Contrast Sensitivity of Human Peripheral Vision

By JYRKI ROVAMO, VELJO VIRSU and RISTO NASANEN. *Institute of Physiology and Department of General Psychology, University of Helsinki, Finland*

Hillz and Cavonius (1974) found that photopic contrast sensitivity of human vision decreases as the gratings are moved farther away from the fovea. Similarly the area of visual cortex devoted to the analysis of a constant size region in the visual field diminishes progressively for more peripheral locations (Cowey and Rolls 1974). In the following we shall provide evidence that decrease of sensitivity is due to diminished cortical area.

We measured the spatial contrast sensitivity functions (CSFs) at various eccentricities along the nasal and inferior meridians of the visual field by finding the inverses of threshold contrasts for a series of sinusoidal gratings. In this experiment the semicircular grating with 8 cm radius was viewed at a constant distance of 458 cm. Eccentricity refers to the angular distance between the fixation point and the nearest edge of the grating. At increasing eccentricities both contrast sensitivity and maximal resolution of high-contrast gratings decreased radically. In the second experiment the eccentricities were the same but both

TABLE I. Adipose tissue blood flow (ATBF), plasma glycerol and core temperature during exercise and induced hyperthermia

	Control	I	II	III	IV	Control
<i>in</i>						
at. ATBF						
$100 \text{ g}^{-1} \text{ min}^{-1}$	1.4 (0.9)	4.0 (1.5)	4.3 (2.1)	5.8 (2.9)	4.8 (2.3)	3.8 (2.3)
per. ATBF						
$100 \text{ g}^{-1} \text{ min}^{-1}$	2.3 (3.2)	4.3 (2.4)	8.1 (4.8)	12.8 (6.3)	15.4 (13.2)	8.1 (3.8)
pl. glycerol						
$\mu\text{mol l}^{-1}$	86 (23)	244 (11)	380 (34)	495 (90)	579 (118)	375 (180)
temperature						
$^{\circ}\text{C}$	36.9 (0.3)	37.8 (0.4)	37.8 (0.2)	37.6 (0.3)	37.3 (0.5)	36.5 (0.2)
<i>reformed</i>						
at. ATBF						
$100 \text{ g}^{-1} \text{ min}^{-1}$	2.3 (1.5)		3.5	(1.2)		1.8 (1.3)
pl. glycerol						
$\mu\text{mol l}^{-1}$	77 (11)		95	(28)		105 (16)
temperature						
$^{\circ}\text{C}$	<36.9		38.5 (0.3)			37.1 (0.3)

Subjects participated in each experimental series. Mean values are given. Figures in brackets are standard deviations.

to study the possible role of the increased subcutaneous ATBF in the regulation of temperature. 2 series of experiments were carried out: A experiments comparing subcutaneous and perirenal ATBF during exercise based on the thought that a possible rise in perirenal ATBF hardly could serve a thermoregulatory purpose. B Measurements of subcutaneous ATBF during passively increased body temperature

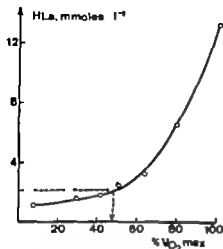
Methods: Subcutaneous ATBF was measured as described in (1). Perirenal ATBF was measured by ^{127}Xe wash-out from the infrarenal fat tissue after injection guided by ultrasound. In series A the subjects worked at 50% maximal O_2 -uptake for 4 consecutive 50 min periods, separated by 10 minute rest intervals. In series B the body temperature was raised about 1.5°C by a hot bath and maintained by stay at an ambient temperature 37°C , humidity 70%. Core temperatures were measured by telemetry using a thermometer swallowed by the subject.

Results and discussion. The experimental findings are given in table I. The increase in subcutaneous ATBF during exercise was confirmed. However the perirenal ATBF increased even more (the increase significant at the 2 percent level). No significant rise in ATBF was seen during passive rise of the core temperature. It is concluded that the rise in ATBF during exercise does not serve thermoregulatory purposes. The increase in ATBF in the two tissues studied correlates well with the intensity of lipolysis as evaluated by plasma glycerol concentrations.

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A.



B.

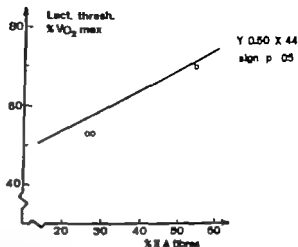


Fig. 1 A. Individual work load - blood lactate curve. The dotted line and the arrow indicate the establishment of the lactate threshold. In this case 48% of $\dot{V}O_{2\max}$. B. The relationship between the percentage of type II A fibres and the lactate threshold ($N=17$)

constructed (Fig. 1 A) and used to determine a so-called lactate threshold (arbitrarily defined as the load in % of \dot{V}_{\max} which is associated with $HLa=2.2$ mM). The range of lactate thresholds was 42-81% $\dot{V}O_{2\max}$. When correlating the lactate thresholds with the percentage values for the different fibre types a significant relationship was observed for type II A only (Fig. 1 B).

It is known that HLa at a given relative load decreases after training. Further after training the number of type II A fibres increases at the expense of type II B (Andersen & Henriksson). The results of the present study suggest that the two phenomena are causally related.

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D 29

Subcutaneous and Perirenal Adipose Tissue Blood Flow during Prolonged Exercise in Man
 By J BÜLOW and J MADSEN *Institute of Medical Physiology C University of Copenhagen Denmark*

It has previously been shown (1) that subcutaneous adipose tissue blood flow (ATBF) in man increases 3-4 fold during prolonged exercise. The purpose of the present experiments

light (BW-TBW) and increased their metabolic rate while some subjects responded aporetic. The individual subjects showed the same pattern during semistarvation i.e. either a decrease in the metabolic rate and a constant weight or vice versa. The changes in metabolic rate in each subject were of the same magnitude regardless of the conditions i.e. rest or work, before or after a meal. The observed change in metabolic rate if supposed to be present all 24 hours of the day only accounts for about one tenth of the increased or decreased dietary intake in the subject with constant weight. The experiments have no explanation for this discrepancy

D 31

Effects of Glucagon and Secretin on Bile Production in Cats

By N. KRABT and J. A. LARSEN *Institute of Physiology University of Aarhus*

Although similar in structure the biological effects of glucagon and secretin differ in many respects. In the present experiments the effects of the two hormones on bile production and liver metabolism were studied in fasting cats anaesthetized with chloralose. Bile was collected from the choledochus and the biliary loss of bile acids was compensated for by continuous infusion of taurocholate. The canalicular bile production was estimated by the biliary clearance of ^{14}C -erythritol. After a control period of 60 min glucagon ($0.1 \mu\text{g/kg/min}$) or secretin ($2 \mu\text{g/kg/h}$) was infused. After a 45 min delay glucagon increased bile flow by 30% due to an increase in canalicular bile production. The bile acid excretion rate remained constant and HCO_3^- -excretion rate increased in parallel with bile flow. Infusion of secretin was immediately followed by a 84% increase in bile flow caused by a change from net reabsorption to net secretion of fluid in bile ductuli. The bile acid excretion rate was not affected but in contrast to glucagon secretin caused a 156% increase in the excretion rate of HCO_3^- . It thus appears that although both hormones do increase bile flow the mechanism of action is quite different, glucagon affecting the non-bile-acid dependent canalicular bile production and secretin stimulating the bile ductuli secretion of a bicarbonate rich fluid.

D 32

β -adrenoceptors Mediate the Stimulating Effect of Adrenaline on Active Electrogenic Na^+ - K^+ -transport in Rat Soleus Muscle

By J. A. FLATMAN and T. CLAUSEN *Institut of Physiology University of Aarhus DK-8000 Aarhus C Denmark*

It has been known for a long time that catecholamines (CA) can hyperpolarize skeletal muscle fibers (see Bowman and Nott 1969). Tashiro (1973) demonstrated that isoprenaline increases the membrane potential in guinea pig soleus muscle, and that this effect was blocked by ouabain.

Energy Expenditure and Weight Responses to Overeating and Semistarvation in Man

By O LAMMERT A G MØLGAARD E S HANSEN and M G RASMUSSEN *Inst for Physical Education Odense University Denmark*

The effect of a variation in the dietary intake on the oxygen uptake the respiratory quotient the body weight and total body water was investigated. The results were compared with measurements obtained during a preceding period with a normal habitual food intake. Before and after a testmeal (4 MJ) $\dot{V}O_2$ and $\dot{V}CO_2$ were measured at rest and at work (36 W). The weight of the dry body matter was determined from the body weight (BW) and determination of total body water (TBW) (tritium).

Measurements were performed in nine normal subjects once a week while dietary energy intake was normal and habitual and while it was increased to 24 MJ per day for two weeks. Four of the subjects continued another two weeks with an intake of 2.1 MJ per day. The daily dietary intake was weighed and the energy content was determined using tables. The results are shown in figure 1. During overeating some subjects kept a constant

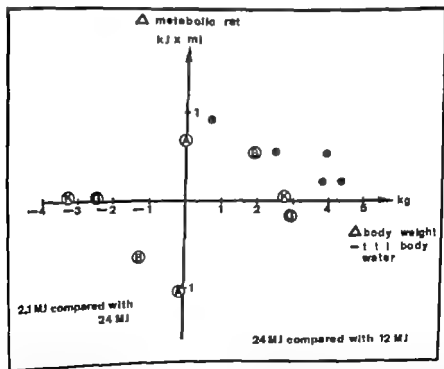


Fig. 1 Changes in energy expenditure in nine normal subjects compared with their changes in dry body matter (body weight - total body water). The right side of the diagram shows the changes obtained when the normal period (12 MJ per day) was compared with the overeating period (24 MJ per day).

The left side of the diagram shows the changes obtained on four of the nine subjects when the overeating period (24 MJ per day) was compared with the semistarvation period (2.1 MJ per day). The letters (A-B-G & K) are used to identify the subjects in the two situations.

In cats an ileal segment was isolated with intact nervous and vascular supply and with ends outside the abdominal wall (Thiry Vella loop). After recovery from surgery the animals were subjected to acute experiments under chloralose anesthesia. The vagi were cut in the neck and stimulated in the efferent direction to ensure a constant and known submaximal level of cholinergic tone in the stomach, the volume of which was recorded by a large balloon inserted via the esophagus. The spinal GI-GI reflex could be repeatedly elicited by distension of the isolated Thiry Vella loop, which elicited a prompt increase in gastric volume. With this preparation it was thus possible to study sympathetically mediated effects induced by hypothalamic stimulation in the presence or virtual absence of spinal GI-GI reflex influences on gastric motility.

Systemic topical stimulations of the hypothalamus revealed an area which could abolish the inhibition of stomach motility caused by the GI-GI reflex, but without affecting gastric tone if this reflex was not present. In addition, stimulation of this area, which appeared to coincide with the hypothalamic sympatho-inhibitory area described by Folkow, Johansson and Öberg (1959) regularly caused decreases in arterial blood pressure and heart rate. It is concluded that there are well-defined hypothalamic sympatho-inhibitory neurons which exert an inhibitory influence on the spinal GI-GI reflex, in association with widespread inhibition of sympathetic discharge to the cardiovascular system.

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D 34

Insulin and Bile Production in the Cat

By J. A. LARSEN, O. ØSTERGÅRD THOMSEN and H. ØRSKOV, *Institut of Physiology, University of Aarhus and Second University Clinic of Medicine, Kommunehospitalet Aarhus*

Previous experiments have demonstrated that administration of insulin stimulates that part of the canalicular bile production which seems to be dependent on the activity of Na-K-ATPase (Larsen and Christensen 1977). Whether this effect of insulin is a direct effect of the hormone or secondary to metabolic changes caused by insulin is not established. In order to examine if insulin actually comes into contact with the canalicular membrane the biliary excretion of insulin was examined. The experiments were performed on 6 fasting cats anesthetized with chloralose. Insulin (Insulin Leo® Neutral, Nordisk Insulin Lab) was given i.v. in doses ranging from 0.15 U/kg to 1 U/kg. Bile was collected from the choledochus and the loss of bile acids compensated for by infusion of taurocholate 0.2 mmole/kg/min. Canalicular bile flow was estimated by the biliary clearance of C¹⁴-erythritol. Insulin in arterial and portal venous blood and bile was determined by a double-antibody technique using Wick chromatography (Ørskov, Thomsen and Yde 1968).

In a combined electrophysiological and ionic flux study we have shown that in the isolated rat soleus muscle CA at concentrations down to the physiological level induce a hyperpolarization which is presumably the result of stimulation of active coupled Na⁺ K⁺-transport (Clausen and Flatman 1977). Both actions were potentiated by theophylline and could be mimicked by dibutyryl-cyclic AMP. The effects of adrenaline were blocked by the β_1 - β_2 -adrenoceptor antagonist propranolol but were unaffected by the α -adrenoceptor antagonist thymoxamine. Moreover, as the specific β_1 -adrenoceptor agonist salbutamol was found to be a potent stimulator of active electrogenic Na⁺ K⁺ transport, we concluded tentatively that these actions of CA were mediated by β_2 -adrenoceptors.

We have now found that adrenaline is at least 100 times more potent than the specific β adrenoceptor agonist H 133/22 at stimulating active Na-extrusion and evoking hyperpolarization in rat soleus muscle. In the heart, adrenaline is 10 times more potent than H 133/22 at stimulating β_1 -adrenoceptors (E. Carlsson *pers. comm.*). Propranolol (10^{-7} – 10^{-8} M) is at least 10 times as potent as the specific β_1 -adrenoceptor antagonist metoprolol at inhibiting the action of adrenaline (10^{-7} – 10^{-8} M) on ^{22}Na -efflux and suppressing the hyperpolarizing effect of adrenaline. As propranolol and metoprolol are nearly equipotent at blocking β_1 -adrenoceptors (Åblad *et al.* 1973) our results provide further support for the idea that the effect of CA on the electrogenic Na⁺ K⁺ transport in rat soleus muscle is mediated via β_2 -adrenoceptors.

We thank Drs. B. Åblad and E. Carlsson of AB Hässle for stimulating discussion and the gift of metoprolol and H 133/22.

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D 33

Hypothalamic Inhibition of the Spinal Gastrotestino-Gastrointestinal Reflex

By D. DELBRO, K. BONGSELL and B. LISANDER. *Department of Physiology, University of Göteborg, Sweden*

Both motility and circulation in the gastrointestinal tract are modulated by the gastro-intestino-gastrointestinal (GI-GI) reflex whose efferent link is the regional adrenergic outflow. This reflex can be elicited by distension of the gastrointestinal tract or by nociceptive stimuli within the abdominal cavity. Although basically spinal it is subject to a supraspinal modulation which is net inhibitory in nature (*cf.* Furness and Costa 1974). The present experiments have been performed to elucidate whether this reflex can be influenced from specific areas of the hypothalamus.

found that 6-OHDA treatment caused considerable reduction of the adrenergic but single terminal axons did persist between the two media layers. No marked change in muscular dimensions was observed. Functionally some phasic spontaneous activity was recorded in all preparations although in sympathectomized tissues it did not persist throughout the experiments. The sensitivity to exogenous noradrenaline (NA) increased in 6-OHDA treated vessels and the maximum response was greater than in controls. Maximum responses to transmural nerve stimulation (NS) were reduced when expressed as a percentage of the maximum NA response (59 ± 3.5 v.s. 89 ± 1.5 per cent $n=6$). Furthermore significant responses to NS were only obtained at high "unphysiological" impulse rates in 6-OHDA treated animals.

concluded that neonatal 6-OHDA treatment leads to marked but incomplete sympathectomy. Growth and morphological differentiation of the media of the portal vein did not occur in treated animals but some functional changes were observed. Apparently no effector nerve control persisted. This would indicate either that the sparse remaining adrenergic supply had been sufficient to govern ontogenesis or that development was controlled by other mechanisms.

Supported by Swedish Medical Research Council 2207 4173 3884 Magn. Bergvall's donation and the Medical Faculty

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D 36

Optic β -adrenoceptor Mediated Facilitation of Noradrenaline Release in Rat Portal Veins

C. DAHLÖF, B. LJUNG and B. ÅBLAD *Dept of Pharmacology AB Hälsjö Mölndal, Sweden*

1) superfused portal veins of spontaneously hypertensive rats (SHR) were used in an attempt to quantify the importance of a presynaptic β -adrenoceptor mediated feedback mechanism for neuronal noradrenaline release and effector response (Adler-Graschinsky 1974 Dahlöf *et al.* 1975 Skjærne *et al.* 1975). The veins were preincubated in Krebs solution containing $(8.5 \times 10^{-7}$ M) 3H 1-noradrenaline (NA) at $+37^\circ C$. After rinsing, the vein preparation was mounted in an organ bath with continuous flow of Krebs solution $(1.5 \text{ ml} \times \text{min}^{-1})$ at a passive tension of 5 mN. Muscle tension was recorded continuously and the effector response (ER) was determined as integrated isometric force. Perfusate was collected in 4 min fractions during periods of spontaneous activity or evoked responses to transmural field stimulation (TNS) with trains of 240 pulses s^{-1} . Three stimulation periods S1, S2 and S3 were performed with 20 min intervals. The fractional release of tritium per shock (Ar) and ER during S3 expressed as a

In accordance with previous experiments insulin increased canalicular bile flow up to 63 % without affecting the excretion rate of bile acids which was 0.30 ± 0.03 $\mu\text{mole/kg/min}$ before insulin and 0.30 ± 0.02 $\mu\text{mole/kg/min}$ after insulin. The insulin concentrations in arterial and portal venous blood were almost identical and after reaching a peak concentration ranging from 235 to 5000 uU/ml they decreased exponentially within 30–90 min. Insulin appeared in bile with a delay of about 15 min with peak concentrations ranging from 40–210 uU/ml and the exponential decay lasted from 30 to 120 min. The occurrence of insulin in bile was always accompanied by a rise in bile flow and changes in the biliary concentration of insulin were often followed by parallel changes in bile flow. In general the insulin concentrations in bile returned to control values before bile flow had normalized.

In conclusion the experiments have demonstrated that insulin is able to pass the canalicular membrane and the possibility therefore exists that the choleretic effect of insulin may be a direct effect of this hormone on the canalicular ATP-ase system.

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D 35

Neuroeffector Development in the Rat Portal Vein after Neonatal Treatment with 6-hydroxy dopamine

By B. LJUNG, J. LUNDBERG and A. DAHLSTRÖM. *Department of Physiology and Institute of Neurobiology, University of Göteborg, Sweden*

During the first few postnatal weeks the media of the rat portal vein becomes divided into a heavy outer longitudinal and an inner circular layer. A terminal adrenergic nerve plexus forms between the muscle layers (Lundberg *et al.* 1976). Correlated to this morphological maturation, functional properties develop in sequence. During the third week of life phasic spontaneous activity and responses to nerve stimulation at low frequencies appear, i.e. characteristic features of the adult portal vein (Ljung and Stage 1975).

With the aim of elucidating the role of the adrenergic innervation for the ontogenesis of propagating vascular muscle, we have studied effects of adrenergic denervation induced by intense 6-hydroxydopamine (6-OHDA) treatment on morphological and functional development of the rat portal vein.

Newborn Sprague Dawley rats were treated with 6-OHDA (100–250 mg/kg s.i.) dissolved in saline with ascorbic acid or with ascorbic acid solution repeatedly during the first two postnatal weeks. At 4 weeks of age the rats were sacrificed and isolated portal veins were either studied directly by the fluorescence histochemistry method of Hillarp and Falck or mounted in an organ bath for isometric recording of contractile activity of the longitudinal muscle and thereafter studied histochemically as stretch preparations.

in found that 6-OHDA treatment caused considerable reduction of the adrenergic but single terminal axons did persist between the two media layers. No marked change in muscular dimensions was observed. Functionally some phasic spontaneous activity was recorded in all preparations although in sympathectomized tissues it did not persist throughout the experiments. The sensitivity to exogenous noradrenaline (NA) decreased in 6-OHDA treated vessels and the maximum response was greater than control. Maximum responses to transmural nerve stimulation (NS) were reduced when expressed as a percentage of the maximum NA response (59 ± 3.5 v.s. 89 ± 1.5 per cent $p < 0.01$). Furthermore significant responses to NS were only obtained at high "unphysiological" impulse rates in 6-OHDA treated animals.

It is concluded that perivascular 6-OHDA treatment leads to marked but incomplete sympathectomy. Growth and morphological differentiation of the media of the portal vein did not persist in treated animals but some functional changes were observed. Apparently no effect of transmural nerve control persisted. This would indicate either that the sparse remaining adrenergic supply had been sufficient to govern ontogenesis or that development was controlled by other mechanisms.

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D 36

Synaptic β -adrenoceptor Mediated Facilitation of Noradrenaline Release in Rat Portal Veins

By C. DAHLÖF, B. LUND and B. ÅRLAND, *Dept. of Pharmacology AB Hässle Mölndal, Sweden*

Isolated and superfused portal veins of spontaneously hypertensive rats (SHR) were used in an attempt to quantify the importance of a presynaptic β -adrenoceptor mediated feedback mechanism for neuronal noradrenaline release and effector response (Adler-Graschinsky 1974 Dahlöf *et al.* 1975 Stjärne *et al.* 1975). The veins were preincubated in Krebs solution containing (8.5×10^{-7} M) ^3H -1-noradrenaline (NA) at $+37^\circ\text{C}$. After rinsing, the portal vein preparation was mounted in an organ bath with continuous flow of Krebs solution ($1.5 \text{ ml} \times \text{min}^{-1}$) at a passive tension of 5 mN. Muscle tension was recorded continuously and the effector response (ER) was determined as integrated isometric force. Effluent superfusate was collected in 4 min fractions during periods of spontaneous activity and during neurogenic responses to transmural field stimulation (TNS) with trains of 240 pulses at 10 Hz. Three stimulation periods S1, S2 and S3 were performed with 20 min intervals. The effect was expressed as fractional release of tritium per shock (Δt) and ER during S3 expressed as a

percentage of those obtained during S2 were used to evaluate the effects of the test substances which were introduced 16 min before S3

It was found that tetrodotoxin (8×10^{-7} M) abolished both Δt and ER and phenoxybenzamine (10^{-6} M) abolished ER. Together these findings indicate that ER was due to neurotically released NA

Phenoxybenzamine produced an almost 5 fold increase of Δt probably mainly due to inhibition of presynaptic α receptors mediating a negative feedback of neuronal NA release (Starke 1972)

Isoprenaline (3×10^{-6} M) increased Δt by 12 ± 3 per cent ($p < 0.05$) but did not affect the ER.

dl-propranolol (5×10^{-6} M and 5×10^{-7} M) caused a dose dependent reduction of Δt by 9 ± 2 ($p < 0.05$) and 31 ± 5 per cent ($p < 0.001$) respectively ER was not affected

d-propranolol (5×10^{-7} M) had in contrast to dl-propranolol no effect on Δt . Since d-propranolol has the same membrane stabilizing action as dl-propranolol but much less β -adrenoceptor blocking potency the findings show that the reduction in transmitter release in response to dl-propranolol was due to β -blockade

Despite the increase in transmitter release during exposure to isoprenaline and the reduction in transmitter release during β -blockade the ER was unchanged. This is an expected consequence of the narrow neuromuscular gap in the portal vein where transmitter concentration peaks of supramaximal amplitudes are obtained with each nerve impulse (Ljung 1976)

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D 37

Role of Blood-borne Catecholamines for Plasma Volume Restoration in Haemorrhage

By J. HILLMAN and J. LUNDVALL. *Department of Physiology University of Lund Sweden*

Mobilization of fluid from the extravascular space of skeletal muscle into the circulatory system is an important mechanism for plasma volume restoration in haemorrhage. This absorption is to a great extent caused by decrease of capillary hydrostatic pressure (P_c) due to sympathetically induced increase of pre- to postcapillary resistance ratio (ra/rv). The present experiments on cat skeletal muscle indicate that the decrease of P_c mainly is mediated by the sympathetic vasomotor fibers in the early period of haemorrhage and

ter is mainly linked to release of catecholamines from the adrenal glands. Activation of the β -adrenoceptors in the microcirculation seems essential for the fluid absorption.

In the superfused innervated muscle standardized haemorrhage (15% of the blood volume) evoked an initial, rapid fluid absorption which gradually declined to attain within less than 5 min a steady rate, maintained during the period of observation (10-15 min). The steady state absorption was much reduced or abolished after adrenalectomy as well as after regional blockade of the β -adrenoceptors. In the cross-circulated muscle haemorrhage of the recipient caused a similar initial absorption as described in the autoperfused muscle. However this absorption was markedly attenuated or even ceased within 5 min despite maintained increase of regional resistance.

The cross-circulation experiments suggest that nerve mediated transcapillary fluid absorption in bleeding, although quite significant, only occurs during a limited period of time. This is in accordance with experiments using artificial sympathetic nerve stimulation and may be due to interference from local vascular control systems causing gradual decline of the α -adrenoceptor induced increase of precapillary resistance and return of ra/rv to control level. The importance of the adrenals for the maintenance of the steady state fluid flow into the circulatory system seems mainly related to activation of β -adrenoceptors. The β -adrenergic effect could be ascribed to dilatation of the precapillary sphincters leading to an increased number of patent capillaries available for transcapillary fluid exchange, and to decreased P_c . Such a β -adrenergic effect on P_c must imply an increase of ra/rv apparently due to a resistance vessel dilatation which, in relative terms, is more pronounced in the post- than in the precapillary vessels.

D 38

Saline Permeability Properties of Myelinated Nerve Fibres from the Rat. Potential Clamp Experiments

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The permeability properties of the nodal membrane in myelinated nerve fibres from albino rats were investigated with potential clamp technique. The method was similar to the one earlier developed for the analysis of frog myelinated nerve fibres (Dodge and Frankenhaeuser 1958). Single large fibres (10-12 μm) were isolated by microdissection from the sciatic nerves. This procedure was more difficult in the rat compared to the isolation of frog nerve fibres. The potential clamp analysis was carried out on fibres that at a resting potential of -70 to -80 mV (set by the d.c. balance control of the feed-back amplifier) had action potentials of about 100 mV and a threshold of 15 to 20 mV.

Records were made of the membrane current associated with different potential steps. At positive test pulses there was a transient initial inward current which depended on external $[K^+]$ and reversed direction at large positive steps. The current density was calculated from estimates of the nodal area (22.5 μm^2) and the internodal impedance (32.4 M Ω)

in 12 μm fibres. The amplitude of the Na current was affected by the potential preceding the test pulse. Similar to the steady state inactivation (h_∞) curve in frog nerve (Frankenhaeuser 1960) a smooth symmetrical sigmoidal curve described the change in peak Na current at different conditioning potentials. The time course of the Na inactivation process was measured (in three fibres) by varying the duration of the conditioning pulse. It was exponential with a time constant that was maximal (5–7 ms) at potentials close to resting potential and minimal (0.5–1.0 ms) at large negative and large positive conditioning pulses. The following quantitative data (mean values) were extracted from measurements at 22–25°C in four fibres: Na current reversal potential 44 mV, maximum peak P_{Na} (constant field Na permeability) at large positive steps $4.6 \times 10^{-8} \text{ cm s}^{-1}$, potential at which P_{Na} is 50% turned on –43 mV, h_∞ at resting potential 0.68.

These results are in agreement with an earlier description of the Na current versus potential relation in rat nerve (Horácková, Nonner and Stämpfli 1968). The Na permeability properties of myelinated nerve fibres have earlier been analysed in potential clamp experiments in frog nerve (cf. Frankenhaeuser and Huxley 1964). The present data indicate that rat and frog myelinated nerve fibres have essentially similar Na permeability properties.

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D 39

Copper Induced Abnormal Chromatin Stability in Human Ejaculated Spermatozoa

By U. KVIST and R. ELIASSON. *Reproductive Physiology Unit, Department of Physiology, Karolinska Institutet, S-10401 Stockholm 60, Sweden*

The nuclear chromatin condenses during spermiogenesis and achieves a high degree of stability when the spermatozoa pass through the male genital tract. The stabilizing process will normally be completed at the time of ejaculation by components in the seminal plasma. In the female genital tract the chromatin is then destabilized and at the time of fertilization there is a rapid decondensation, necessary for the subsequent cell divisions. Therefore condensation and decondensation of the nuclear chromatin of the spermatozoa are processes of importance for fertility. The role of various endogenous and exogenous factors on these processes are under investigation. In this report the in vitro effects of copper on ejaculated spermatozoa will be presented.

The chromatin of human spermatozoa is normally stabilized by zinc interacting with

groups. The degree of stability can be measured as resistance to various agents, e.g. sodium dodecyl sulphate (SDS). Ejaculated spermatozoa are normally stabilized and do not decondense when exposed to SDS alone but do decondense when treated with both the chelating agent e.g. EDTA (2 mM) and SDS (SDS-EDTA) (Kvist U To be published).

Cu^{2+} (1 mM) can induce chromatin stability by oxidation of thiol groups into disulfide bridges (Bedford J. M. et al. J. Reprod. Fert. 1973 33 19). We have found that Cu^{2+} (1.6 mM) induced a high degree of stability towards both SDS and SDS-EDTA. Furthermore, copper releases zinc from the spermatozoa.

As copper will not only increase chromatin stability but also change the physiological zinc dependent stability into a different and probably abnormal, non-zinc dependent stability. Oxidation of thiol groups into disulfide bridges in the nuclear proteins and a consequent displacement of zinc from these thiol groups seems to be a plausible explanation for our results.

Physiological destabilization by zinc release from the spermatozoa in the female genital tract seems to be possible since follicular fluid can induce zinc release in vitro (Johnston and Eliason, R. Andrologia 1976, 8 283). In addition to copper we have found other oxidative agents and in vitro storage of the spermatozoa change the normal stability into disulfide bridge dependent stability. Such chromatin may not destabilize in the female genital tract. It is known that aged spermatozoa have a reduced fertilizing ability and give rise to a high incidence of fetal abnormalities. Further studies how various factors can influence the chromatin stability in human spermatozoa are in progress.

D 40

Adenosine Effects on Cholinergic Neurotransmission in the Rabbit Stomach in vivo

By L. GUSTAFSSON, P. HEDQVIST, B. B. FREDHOLM and S. ÖLUND. *Departments of Physiology and Pharmacology Karolinska Institutet Stockholm Sweden*

There is evidence that adenosine inhibits the release of acetylcholine in the guinea pig in vitro (Vizi and Knoll 1976, Sawynok and Jhamandas 1976, Gustafsson et al. 1977). The presynaptic effect of adenosine is inhibited by methylxanthines and enhanced by agents that inhibit adenosine inactivation, such as dipyrindamol and diltiazem. The present study was undertaken to investigate whether a presynaptic effect of adenosine may be demonstrated also in vivo.

Rabbits anaesthetized with sodium pentobarbitone were used. The left and/or right vagus was cut and stimulated in the neck-region. Gastric motor responses were registered by means of a strain gauge transducer. Blood pressure, heart rate and pulmonary insufflation pressure were also recorded. A thin plastic indwelling catheter was placed in the celiac artery. Drugs (acetylcholine, adenosine, diltiazem) were administered either via the celiac artery or into the left ventricle.

Electric stimulation of 1 min of the vagus (3-5 Hz, 5-8 V, 1 msec duration) gave re-

producible contractile responses in the stomach and reductions of heart rate without significant alterations in blood pressure. The vagal effects were inhibited by atropine. Acetylcholine infusion (3° -640 μ g/min) into the coeliac artery produced clearcut contractile effects in the stomach with minimal effects on blood pressure or heart rate.

Adenosine given into the coeliac artery (0.5-1 mg/min) did not produce any effects on blood pressure or heart rate while the same dose given into the heart typically reduced blood pressure from 80 to 30 mmHg. However, when the adenosine uptake inhibitor dilazep (0.25-1 μ mol/kg) had been given, intraarterial adenosine produced pronounced systemic effects. These results demonstrate the presence of a very active adenosine removal system which is inhibited by dilazep.

Adenosine infusion into the coeliac artery inhibited the gastric response to vagus stimulation by 50% or more. This effect of adenosine was enhanced by dilazep. On the other hand, adenosine enhanced the contractile response to intrarterial acetylcholine. The fall in heart rate following vagus stimulation was not inhibited by adenosine.

The present results are compatible with the opinion that also *in vivo* adenosine might inhibit acetylcholine release from cholinergic nerve endings. In addition, adenosine might potentiate some postjunctional effects of acetylcholine by an unknown mechanism. A similar differential effect of adenosine on pre- and postjunctional components in adrenergic neurotransmission has been described earlier (Hedqvist and Fredholm 1976).

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D 41

In Vitro Studies of Antler Blood Vessels

By M. WIKÅ, *Dep. anat. Dent. Fac. Univ. Oslo, Norway* and L. EDVINSSON, *Dep. histol. Univ. Lund, Sweden*

Antler development and function have been a matter of much debate. Antlers have for some time been regarded as having thermoregulatory function. Thermoregulation is coupled with the vascular system and may involve amine receptors. A study on innervation of the antler vascular bed has shown well innervated vessels below the antler and in all but a few occasions non-innervated vessels in the antler. Antler arteries have a thick muscular wall with a longitudinally directed inner layer and a circularly oriented outer layer. The adventitia contains mast cells which may influence circulation.

Pieces of blood vessels from domestic reindeer were studied for circular and longitudinal muscle activity in an *in vitro* system. The vessels were kept in a ring on ice and were found to be viable for several days.

supplying arteries below the antler show circular muscle activity which may be initiated even in the set up for registration of longitudinal tensions, as an increase in the vessel piece. Antler arteries contract both in circular and longitudinal directions. Pieces of the vessels contract in a dose dependant way upon exposure to noradrenaline, phenylephrine, histamine and 5-HT. The alpha adrenergic type receptor can be demonstrated for noradrenaline, adrenaline and phenylephrine by using phentolamine, phenoxybenzamine and dibenamine as blocking agents.

Cells of antler blood vessels have further been found to be spontaneously and rhythmically active. 11 of 4 pieces tested for circular activity and 11 of 22 tested for longitudinal activity showed vigorous variable rhythmical contractions. In some cases this type of activity was elicited by pharmacological agents as PGP_{12/13} or small concentrations of catecholamines.

So far we have not been able to clearly demonstrate adrenergic beta receptors in antler blood vessels.

The spontaneous rhythmic activity in antler blood vessels is in good agreement with our findings of an oscillating blood flow to the antler when measured by electromagnetic flowmetry (M. Wik & J. Krog in progress). In these measurements the vascular bed of the antler part of it seem to go into spasms that reduce or completely stop the antler circulation for a considerable length of time. The reason for this could in certain cases be manipulations, but in other cases the causes could not be identified.

D 41

Effect of Intracellular Ca^{++} on Pinocytosis

by P. JOHANSSON and J.-O. JOSEFSSON, Department of Pharmacology, University of Lund, Sweden

Calcium in the extracellular medium greatly influences the formation of pinocytotic channels in *Amoeba proteus*. Micromolar concentrations of Ca^{++} stimulate while millimolar concentrations inhibit cation (Na^+) induced pinocytosis. The inhibitory effect reflects competition between the inducing cation and Ca^{++} at the cell surface while the stimulating effect of Ca^{++} is exerted on some channel-forming apparatus in the cytosol (Josefsson, 1975).

In addition to these effects of Ca^{++} the following observations on pinocytosis in Ca^{++} media indicate that passage of Ca^{++} from the cell surface into the cell or ionization of Ca^{++} in the cell interior may inhibit pinocytosis:

Agents which activate or increase the capacity of the cell for pinocytosis (e.g. 10^{-6} M bAMP, 10^{-6} M theophylline, 2 mg/l ionophore A 23187, 10^{-6} M noradrenaline, 10^{-6} M verapamil) inhibit the formation of pinocytotic channels when added to the Ca^{++} -free incubation medium. Neither inhibition nor activation occurs in calcium-depleted cells (cells treated with Ca^{++} -deficient media). None of the activating agents with the exception of A 23187 is inhibitory in the presence of 10^{-6} M verapamil.

2 Caffeine (0.2 mM) inhibits pinocytosis in normal cells but not in Ca^{++} -depleted cells. Procaine (10^{-4} M) or high Mg^{++} antagonizes the effect of caffeine. Inhibition by caffeine is potentiated by activating agents but not affected by verapamil.

3 Substitution of Cl^- in the inducing solution for a monocarboxylate anion (propionate, acetate, lactate, glycolate) decreases pinocytosis intensity. The degree of blockade increases with the pK of the acid and the calcium content of the cell. The mechanism of inhibition is probably release of Ca^{++} from intracellular stores. Caffeine, cAMP (10^{-4} M) and activating hormones potentiate blockade of pinocytosis by acetate, while cGMP (10^{-6} M), insulin (10^{-6} U/l) and somatostatin (10^{-6} g/l) restore normal pinocytosis.

4 Pinocytosis inhibited by Ca^{++} -depletion is normalized by activating agents, caffeine or acetate but further impaired by insulin, somatostatin or verapamil.

In conclusion, pinocytosis intensity increases when the free Ca^{++} concentration of the cytosol attains a certain level but decreases when Ca^{++} exceeds that level. This dual effect of Ca^{++} may contribute to the understanding of the transient character of cation-induced pinocytosis, the extremely weak inducing potency of calcium salt solutions and the complex shape of the dose-response curves of cationic inducers. Mammalian hormones may either reduce (insulin, somatostatin) or increase (activating hormones) the intracellular free Ca^{++} level and so modify the intensity of pinocytosis in the amoeba.

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D 43

Autonomic Control of Penile Volume in Rabbits

By N. O. SJÖSTRAND and E. KLINGE, *Department of Physiology I, Karolinska Institutet, Stockholm, Sweden and Department of Pharmacology, University of Helsinki, Helsinki, Finland*

The autonomic pathways and mediators behind penile erection and relaxation have been under debate. Partly, this could be due to the circumstance that no one seems to have stimulated the autonomic nerves to the penis with lower frequencies than 70 Hz (vide Klinge and Sjöstrand 1974). Here, some data on the relationship between penile volume and frequency of stimulation of these nerves are presented.

Rabbits anesthetized with urethane were used. Ureters were cannulated. Bladder, jejunum, ileum, colon and upper part of rectum were removed. Vasa deferentia were tied. The penis was skinned and placed in a greased glass plethysmograph. The skin was tied around as sealing. Penile volume was recorded with a low pressure transducer on a Grass polygraph. Hypogastric and pelvic nerves as well as the sympathetic chain (L6-S1) were cut and placed on stimulation electrodes. Supramaximal voltage was used.

Very low frequency stimulation of the sympathetic chain produced profound detumes-

PER CENT OF MAXIMUM RETRACTION OR PROTRUSION

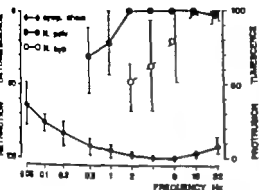


Fig. 1 Frequency-response relationship between penis volume and stimulation of the sympathetic chain and the pelvic and the hypogastric nerves. All nerve stimulations are bilateral. Each point means and range from three expts. Frequency on logarithmic Scale.

ence of the penis (Fig. 1). The resting volume (70-90% retraction) was achieved with 1-0.5 Hz. Stimulation of the pelvic as well as the hypogastric nerves produced tumescence of the penis. However the two nerve sets had different frequency-response curves (Fig. 1). The response to hypogastric nerve stimulation could be affected by scopolamine (0.1-1 mg/kg) in rabbits seemingly devoid of atropinase while that to pelvic nerve stimulation was almost unaffected by the drug.

It is suggested that the penis is supplied with a very efficient vasoconstrictor innervation apparently operating at very low frequencies. The organ is supplied with sympathetic as well as parasympathetic dilator fibres. The former of which might—at least in part—be cholinergic while the latter might chiefly be non-cholinergic.

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D 44

Cumulative Blood Flow during Reactive Hyperemia in Human Cutaneous Tissue

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Consider the experiment where blood flow is stopped at $t=0$, released at $t=1$ and blood flow has decreased to the preocclusion level at time t_2 then cumulative blood flow V_2 , between t_1 and t_2 can be written as:

$$V_2 = \int_{t_1}^{t_2} f(t) dt \quad (1)$$

where $f(t)$ is the perfusion coefficient. Applying ^{133}Xe assuming homogeneous perfu-

sion no recirculation and washout of tracer is only flow limited then the externally monitored decrease in activity at time t , $-dq(t)/dt$ is given as

$$(-dq(t)/dt)/q(t) = f(t)/\lambda$$

where $q(t)$ is the activity at time t and λ is the tissue to blood partition coefficient. Rearranging eq 2 and integrating on both sides gives

$$-\lambda \int_{t_1}^{t_2} d \ln q(t) = \int_{t_1}^{t_2} f(t) dt = V_B \quad (3)$$

Solution of eq 3 gives

$$V_B = \lambda \ln(q(t_1)/q(t_2)) \quad (4)$$

Excess cumulative blood flow $V_{B, \text{excess}}$ during reactive hyperaemia can be calculated as

$$V_{B, \text{excess}} = \lambda \ln(q(t)/q(t)_{\text{pre}}) \quad (5)$$

where $q(t)_{\text{pre}}$ is obtained by retropolating the activity curve versus time from t_2 to t_1 . Relative repayment R equals excess cumulative blood flow divided by blood flow deficit calculated as the product of preocclusion blood flow and duration of vascular occlusion i.e

$$R = V_{B, \text{excess}} / (\lambda \cdot k_{\text{pre}} \cdot t_1) = (\ln(q(t_1)/q(t_1)_{\text{pre}})) / (k_{\text{pre}} \cdot t_1) \quad (6)$$

where k_{pre} is the washout rate constant before vascular occlusion.

Six normals were studied. Cutaneous blood flow was measured dorsally on a finger by the local ^{133}Xe washout technique. A cuff was placed proximally on the finger. Vascular occlusion lasting from 3 to 24 min was induced by inflating the cuff to 250 mm Hg.

Prolonging the duration of vascular occlusion from 3 to 12 min caused an increase in maximum cutaneous blood flow from 54 to 104 ml/100 g min. Ischaemia for 24 min did not cause a further increase in maximum blood flow. $V_{B, \text{excess}}$ (eq 5) increased from 17 ml/100 g to 100 ml/100 g following 3 and 24 min of vascular occlusion respectively. Relative repayment, R (eq 6) was not correlated to duration of vascular occlusion. There was an inverse correlation between relative repayment and preocclusion blood flow.

The results indicate that the hyperaemic response is at least partly due to local changes in concentrations of different metabolites liberated in the tissue. The inverse correlation between relative repayment and duration of vascular occlusion suggests that besides the nutritive demand other factors such as heat regulation has influenced preocclusive blood flow in cutaneous tissue.

D 45

Sleep in Cats after a New 5-HT Uptake Inhibiting Drug—Lu 10-171

By T KOVALA, A LEPPÄVUORI and P PUTKONEN *Institute of Physiology University of Helsinki Finland*

Lu 10-171 is a bicyclic phthalane derivative with potent and specific 5-HT uptake inhibiting properties. In contrast to most tricyclic antidepressants it is practically devoid of NA

uptake inhibiting effects and has low anticholinesterase and antihistaminergic activity (Byrd 1977).

The importance of 5-HT for physiological sleep is established by experiments involving depletion and replenishment, but loading with 5-HT precursors has had variable consequences.

We have studied the EEG stages of vigilance after intraperitoneal injections of 1 and 5 mg/kg of Lu 10-171 in 9 chronically implanted cats. The 16 h mean percentages (\pm SD) of the sleep stages are summarized below.

Table 1

Stage	Control NaCl (N=9)	Lu 10-171 1 mg/kg (N=5)	Lu 10-171 5 mg/kg (N=6)
Arousal (A+D)	30.6 \pm 7.9	25.2 \pm 8.0	23.6 \pm 6.0
Arousal (A)	11.6 \pm 5.4	9.5 \pm 4.3	8.5 \pm 3.0
Drowsy (D)	19.0 \pm 5.6	15.6 \pm 5.4	15.1 \pm 6.4
Slow wave sleep (S1+S2)	33.5 \pm 6.4	42.8 \pm 11.0	74.1 \pm 5.6
Light SWS (S1)	23.8 \pm 3.7	23.2 \pm 8.5	20.7 \pm 5.5
Deep SWS (S2)	29.6 \pm 3.4	39.6 \pm 8.8*	53.4 \pm 7.4**
Paradoxical sleep (PS)	15.9 \pm 4.9	12.1 \pm 3.9	2.3 \pm 1.2**

* $p < 0.05$, ** $p < 0.001$ Difference from control

The main results were: 1) A dose dependent increase in deep SWS 2) Inhibition of PS with the higher dose. Other stages, especially arousal decreased moderately.

The tricyclic antidepressants which inhibit both 5-HT and catecholamine uptake are known as potent suppressors of PS. Lu 10-171 differs from them in having weaker PS inhibiting effects and promoting specifically deep SWS.

Administration of 5-HT precursors has lead to contradictory results including both increase and decrease of PS in man. In the cat (Urdin 1976) 5-HTP moderately inhibited PS, but, at variance with Lu 10-171 increased the synchronized drowsy pattern instead of SWS. Our results, however are more in agreement with Austret *et al* (1976) describing decrease in PS and increase in deep SWS (stages 3+4) in humans after repeated high doses of 5-HTP.

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A Possible Role of Endocytosis in Lysosomal Activation in Dystrophic and Denervated Skeletal Muscle

By R. LIBELIUS *Department of Pharmacology University of Lund Sweden*

Previous studies have shown that a cobra α neurotoxin (*Naja naja siamensis* Mw 782) binds specifically to cholinergic receptors and is taken up intracellularly by mouse skeletal muscle *in vitro* (Libelius 1974). *In vitro* intracellular uptake of toxin (+37°C) was found to be stimulated by cationic proteins e.g. protamine and is believed to occur by endocytosis (Libelius 1975). In addition biochemical and ultrastructural examination showed that protamine caused lysosomal activation with autophagic vacuolation of muscle fibers. Protamine also stimulated intracellular uptake of another macromolecule horseradish peroxidase in mouse skeletal muscle fibers under *in vitro* conditions (Jirmanová et al 1977).

Since endocytosis and lysosomal activation in response to protamine *in vitro* occur concomitantly in skeletal muscle a causal relation between the two phenomena seems possible. Therefore it was of interest to study endocytosis in dystrophic and denervated muscles both of which display increased activities of lysosomal enzymes (Weinstock and Iodice 1969). Endocytosis *in vitro* and *in vivo* was studied by a combination of biochemical, radiochemical and light and electron microscopic methods using ^3H -inulin and horseradish peroxidase as macromolecular tracers.

In vitro experiments showed that the uptake of both ^3H inulin and horseradish peroxidase at +37°C was increased in leg (extensor digitorum longus and soleus) muscles denervated for 6 days and in leg muscles from hereditary dystrophic mice (Bar Harbor 129 strain) when compared with innervated and littermate control muscles. The uptake of i.v. injected horseradish peroxidase measured biochemically was likewise increased in denervated and dystrophic muscles. When horseradish peroxidase was injected i.v. light microscopic examination of the muscles showed that the tracer was present not only in the extracellular space but also as deposits within denervated and dystrophic muscle fibers. Such deposits of horseradish peroxidase which were not found in normal muscle fibers were found by ultrastructural examination to be membrane limited and were located between myofibrils close to labelled transverse tubules. Primary endocytic vesicles containing horseradish peroxidase appeared to originate from the sarcolemma and the transverse tubules. The results suggest an increase in endocytic activity in dystrophic and denervated skeletal muscle with distribution of exogenous tracers into secondary lysosomes.

Endocytosis regardless of what initiates it may constitute an important mechanism of lysosomal activation and therefore of muscle fiber degeneration.

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D 47

Control of Lipolysis and Circulation in Adipose Tissue by Endogenous Adenosine

By BERTIL B. FREDHOLM and ALF SOLLEVI. From the Department of Pharmacology Karolinska Institute Stockholm Sweden

Sympathetic nerves are the most important influence on adipose tissue circulation and lipolysis. In addition there are local mechanisms regulating adipose tissue function. Adenosine seems to be important in this respect. The release of adenosine from isolated cells *in vitro* (Schwabe *et al.* 1975) and from intact adipose tissue by nerve stimulation (Sollevi 1976) was recently reported. Adenosine is a potent inhibitor of noradrenaline induced cyclic AMP elevation and lipolysis in fat cells (Schwabe *et al.* 1975 Fredholm 7), and it inhibits lipolysis due to nerve stimulation *in vivo* both by a prejunctional and postjunctional effect (Hedqvist and Fredholm 1976). The possibility therefore exists that adenosine released during nerve stimulation modulates adipose tissue function by pre- and postjunctional actions. In the present communication results with two drugs that inactivate adenosine are presented.

Canine subcutaneous adipose tissue with intact blood and nerve supply was studied. The effect of an inhibitor of adenosine uptake, dflazep (Asta Werke Brackwede BRD) and an inhibitor of enzymatic adenosine breakdown, erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA, Wellcome Research Triangle Park, N.C.) on glycerol release and vasoconstriction during nerve stimulation (4 Hz) was studied.

Neither dflazep nor EHNA had any effect *per se* on lipolysis in isolated fat cells (Fredholm 1977). Dflazep (5-20 μ M) caused a 60% reduction in glycerol release induced by nerve stimulation *in vivo* ($n=6$, $p<0.01$) without affecting basal lipolysis. Similar results are reported for a different adenosine uptake inhibitor, dipyrindamol (Fredholm and Sollevi 1977). The maximal vasoconstrictor response to nerve stimulation was inhibited and autoregulatory escape during a 15 min stimulation was enhanced.

EHNA (5-10 μ M) did not alter maximal vasoconstrictor effects but significantly potentiated the autoregulatory escape. Lipolysis was unchanged or even slightly enhanced in parallel with the improved circulation.

Direct measurement of adenosine deaminase showed that the fat cells had only 5% of the total tissue activity although they contribute more than 90% of the cell mass. On the other hand, fat cells had an avid adenosine uptake system with high capacity and an affinity in the μ molar range.

These findings show that different cellular components of the same tissue may inactivate adenosine by different mechanisms. Our results are also compatible with the opinion that endogenous adenosine may be involved in the autoregulatory escape in adipose tissue and as an endogenous modulator of lipolysis.

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II 48

Reflex Adrenergic Control of Pancreatic Hormone Release

By J JÄRIULT S INOEMANSSON and J HOLST *Departments of Physiology and Surgery University of Lund Sweden and Department of Clinical Chemistry Bispebjerg Hospital Copenhagen Denmark*

It has recently been described that unloading of the carotid baroreceptors is associated with a large increase in the blood glucose concentration whereas stimulation of the carotid chemoreceptors had only minor effects on the blood glucose level (Järlhult, Holmberg and Lundvall 1977). It is reasonable to believe that such an hyperglycemic response evoked from arterial baroreceptors also involves an adjustment of the release of the pancreatic hormones and the present study was outlined to test this hypothesis.

The effects of unloading of the carotid baroreceptors on arterial plasma glucose concentration as well as on portal plasma immuno-reactive glucagon (IRG) and insulin (IRI) concentrations were studied in anesthetized vagotomized cats by sectioning of the sinus nerves or in some experiments by lowering the pressure in the isolated carotid sinuses. Cervical vagotomy *per se* slightly decreased the basal IRI concentration and increased the basal IRG concentration whereas plasma glucose remained unchanged. Complete elimination of the carotid baroreceptor discharge by sectioning of the sinus nerves caused a doubling of plasma glucose and an increase in IRG by about 200% whereas IRI decreased to about 50% of its control value. These baroreceptor-induced metabolic and hormonal changes which were maintained during 45 min of observation showed to be mediated mainly by the sympatho-adrenal system since cutting of the splanchnic nerves above the adrenal glands abolished the hyperglycemia and hypoinsulinemia and markedly depressed the hyperglucagonemia. Bilateral adrenalectomy only suppressed the magnitude of these reflex responses whereas sympathectomy of the pancreas virtually abolished them. The pancreatic hormonal adjustments seemed to be graded in relation to the drop in carotid blood pressure and they were clearly detectable when the distending pressure in the carotid sinuses was lowered from 120 to 95 mm Hg. An interesting observation was that i.v. infusion of adrenaline ($1 \mu\text{g/kg} \times \text{min}$) caused hyperglycemia, hyperglucagonemia and hypoinsulinemia whereas infusion of noradrenaline in the same dose raised the IRG concentration twice but did not significantly alter the IRI or glucose concentrations.

We conclude that the release of insulin and glucagon can be strongly influenced by

changes in the arterial blood pressure. This reflex control of endocrine pancreas is derived from arterial baroreceptors and mediated to the gland by the sympatho-adrenal system. The reflex seems particularly dependent on a direct sympathetic innervation of the islets of Langerhans.

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D 49

Effect of Muscle Mass in the Cardiovascular Response to Isometric Contractions

By BENGT SALTIN, J. H. MITCHELL, B. SCHIBYE and E. C. PAYNE. *August Krogh Institute University of Copenhagen Copenhagen Denmark*

During dynamic concentric exercise the cardiovascular response is related to work intensity and muscle mass involved in the work. In contrast in isometric contractions the enhancement of blood pressure (BP) and heart rate (HR) has been shown to be solely a function of the relative exercise intensity and independent of muscle mass involved (Lind and McNicol 1967, McCloskey and Streetfield 1975). When we repeated similar studies we were unable to confirm the independency of the muscle mass for the magnitude of the BP and HR elevation while performing sustained contractions.

Eleven men were studied performing at 40% of maximal voluntary contraction (% MVC) for two minutes with digits II and III (d) handgrip (h) knee extension (k) and combining (b) d and (k). Blood pressure was measured directly (a. brachialis) and recorded together with an ECG and the force output with a Brush recorder. Already early during the contraction the lowest elevation in HR and BP was observed for (d) and the largest for (h) and (k). During the latter phase of the contractions the following values were observed (mean and SD): HR 83.8 ± 12.9 (d) 102.4 ± 14.0 (h) 117.4 ± 19.2 (k) 123.5 ± 18.7 (h+k) BP 153.4 ± 18.7 (syst.) 103.3 ± 12.5 (diast.) (d) 186.4 ± 19.7 128.5 ± 13.8 (h) 170.6 ± 19.6 142.1 ± 12.5 (k) 212.0 ± 29.6 , 153.4 ± 1.5 (h+k). The differences in HR and mean BP comparing the various muscle synergies were significant.

The rather large individual variation in HR and BP responses was due to a certain day to day variation and to the finding of a greater elevation of HR and BP the larger the absolute force developed in the contraction. In fact combining all individual data a close relationship was found for the developed absolute force and the HR and BP responses ($r=0.9$ $P<0.001$). The only explanation we can give for the discrepancy between our and earlier results is that in previous studies the contractions have not been confined to isolated muscle groups and the variation in muscle mass has not been as large as anticipated. To what extent central and peripheral factors contribute in the regulation of HR and BP cannot be stated. By applying cuffs and inflate them to above arterial pressure at the point of termination of the contraction, BP stayed elevated, suggesting a peripheral chemosensitive receptor playing a role in the regulation.

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D 50

Utilization of Dietary Amino Acids by Grouse (*Lagopus Lagopus*)

By A. MORTENSEN and A. TINDALL. *Department of Physiology University of Tromsø Norway*

It has been suggested that the function of the caeca in birds is cellulose digestion (McBee and West, 1969). An alternative idea is that they are important in nitrogen metabolism by the catabolism of some urinary nitrogenous compounds and reutilization of the nitrogen moiety. Barnes (1972) found organisms capable of uric acid metabolism in the caeca of fowl but none capable of digesting cellulose.

We have considered the problem from several angles. 1. We have examined the amino acid content of both artificial and natural foods and of the contents of the ileum and caeca, and also the faeces. 2. We have looked at the absorption of labelled amino acids injected into the caeca and 3. we have done some work on the breakdown of uric acid in the caeca.

Under equithesin anaesthesia, the abdomen was opened and by means of a soft catheter 0.1 mg amino acid mixture (activity = 50 $\mu\text{Ci/ml}$) in a volume of 1 ml was injected being spread throughout a length of several centimetres of one caecum. Blood samples from the wing vein taken at subsequent intervals showed marked rises in radio-activity which indicated absorption from the caecum. It cannot be concluded that such activity detected in the blood is necessarily the labelled amino acids since they may have been catabolized while in the caecum. However the very short time lag between injection and appearance of activity in the peripheral circulation makes this unlikely.

When uric acid is broken down the carbon atoms are normally removed and excreted as CO_2 while the nitrogen-containing portion may be reused after being degraded further even to ammonia. Concentrations of ammonia in the caeca and colon were found to be substantial and in preliminary experiments we have incubated 0.5 g caecal contents with 0.02 ml of a solution of ^{14}C -labelled uric acid (activity = 50 $\mu\text{Ci/ml}$). The released CO_2 was radio-active.

Thus the experiments suggest that either amino acids or their immediate breakdown products are readily absorbed from the caeca. We also think that uric acid is catabolized so that the nitrogen-containing part can be reutilized but further work is required to establish this idea or not.

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D 51

Release of Adenosine-like Material from Isolated "Perfused" Fat Cells

By P. HJENDAHIL, B. B. FREDHOLM and A. SOLLEVI. *Department of Pharmacology Karolinska Institutet Stockholm Sweden*

Adenosine, a breakdown product of adenine nucleotides has been ascribed a role as endogenous modulator of cyclic AMP accumulation and lipolysis in isolated fat cells (cf. Schwabe *et al.* 1975). Adenosine accumulates in fat cell incubates (see Schwabe *et al.* 1975) and is released from adipose tissue *in vivo* (Fredholm 1976). The kinetics of adenosine formation and breakdown are however incompletely known.

³H-adenine or ³H-adenosine was taken up by isolated fat cells and incorporated into nucleosides. The uptake and phosphorylation followed Michaelis-Menten kinetics with a V_{max} of about $2 \text{ nmoles} \times \text{min}^{-1} \times \text{g}^{-1}$ and a K_m in the μmolar range. After labelling the cells were placed in a temperature-controlled plastic chamber and perfused according to Allen *et al.* (1973) with 2 ml buffer/min. This technique allows continuous removal of products formed by the fat cells. The basal rate of release of radioactivity was $0.49 \pm 0.04\%$ of the cellular content per min ($n=11$). Most of the radioactivity released was nucleosides of which adenosine was a major component. Assuming homogeneous labelling of the nucleoside stores the rate of adenosine release is calculated to be $0.3\text{--}0.4 \text{ nmoles} \times \text{min}^{-1} \times \text{g}^{-1}$. The release rate was decreased by $31 \pm 8\%$ ($p < 0.05$) by perfusing with oxygenated rather than aerated buffer. On the other hand, lipolytic stimuli did not significantly alter (a $12 \pm 6\%$ increase) the nucleoside outflow. These findings are in agreement with results obtained *in vivo* and suggest that adenosine release is regulated by oxygen supply and demand but not by lipolysis *per se*.

In contrast to the situation in incubated fat cells adenosine deaminase failed to increase lipolysis in the perfused fat cell system. Exogenous adenosine was more antilipolytic in perfused than in incubated fat cells. These differences may be explained by an accumulation of adenosine in conventional fat cell incubates.

The present results support the contention (Fredholm 1976) that adenosine accumulation in adipose tissue *in vivo* may be caused by blood flow restriction and hypoxia rather than by lipolytic stimulation. The results are also compatible with the opinion that adenosine is an endogenous modulator of lipolysis *in vitro* and *in vivo*.

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D 52

The Effect of Progesterone on the Spontaneous Interictal Spikes Evoked by Topical Application of Penicillin to the Cat's Cerebral Cortex

By S. LANDÖREN, T. BÄCKSTRÖM and G. KALESTRATOV. *Department of Physiology University of Lund, Sweden*

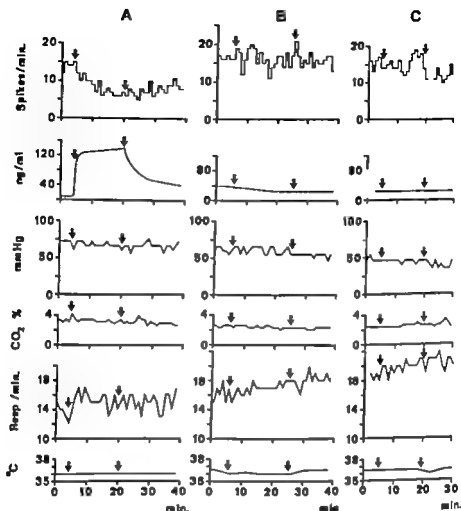


Fig. 1 Columns of graphs (A-C) showing from above downwards: the frequency of interictal penicillin spikes the plasma concentration of progesterone the mean systemic blood pressure PCO_2 respiratory rate and rectal temperature. A. Intravenous infusion of progesterone B. control infusion of normal cat serum C. Infusion of Metbumal 0.11 mg/ml. The graphs were obtained from three different foci A, B and C in the same animal. The chronological order was A B C. The arrows mark the beginning of the priming dose and the end of the infusion.

The frequency of seizures in female epileptic patients is positively correlated to the quotient of estrogen/progesterone in their plasma (Bäckström 1976). Previous investigations indicate that estrogen may have epileptogenic effects. The role of progesterone is, however, less well understood. We have therefore studied the effect of intravenous progesterone infusions of low concentrations on the discharge of a penicillin focus in the cerebral cortex of the ovariectomized cat.

Filter paper (4 mm²) soaked in Na-benzylpenicillin 100 000 IU/ml were placed on the cortical surface near the post cruciate dimple. A focus discharging spontaneous interictal spikes at a quasi-regular frequency was established within 10 minutes. The spikes were recorded from the cortical surface near the focus. Recordings of electrocorticogram as

tidal blood pressure, end tidal PCO_2 , respiratory rate and rectal temperature was made on Sencor Elena Mingograf. Progesterone dissolved in serum from ovariectomized cat or Mebomal (ACO) diluted with physiological saline were injected intravenously. A priming bolus (1.5 ml) was followed by a slow constant infusion (11 ml/h). Blood samples were taken before, during and after the infusion and the plasma progesterone was assayed using a direct radio-immunoassay method described by Carstensen and Bäckström (1976).

The effect of progesterone infusion is shown in Fig. 1. In 10 out of 11 experiments a decrease in the frequency of the spontaneous interictal discharge was observed. Considerable effects were seen with plasma concentrations of progesterone between 40 and 70 ng/ml. Infusion of Mebomal in equimolar doses was less effective. The amplitude of the negative penicillin spike was reduced and the spike disappeared more rapidly during progesterone infusion than in control experiments.

It was concluded that progesterone in physiological plasma concentrations influenced the generation of the interictal penicillin discharge.

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D 53

Durnal Rhythm of Hypothalamic TRF in the Rat

By J. LEPPÄLUOTO, P. KORVUVALO and R. KRAANIA, *Department of Physiology, University of Oulu, Finland*

We have previously shown that the highest serum TSH levels occur in the middle of the light period in rats (Leppälüoto, Ranta and Tuomisto 1974). To determine if these high TSH levels might be correlated with hypothalamic TRF secretion, we have measured TRF contents of rat hypothalami at various times of the light-dark period.

Rats were adapted to a silent room for 2 weeks, lights on from 8 AM to 6 PM. Six animals were decapitated at 4 hrs intervals and hypothalami and various brain parts were rapidly excised, weighed, extracted to methanol and purified in cation exchange chromatography. The eluates were assayed in TRF radioimmunoassay. In male rats higher hypothalamic TRF contents were found during the light than dark period (380 ± 65 vs. 160 ± 66 pg/mg of tissue, $p < 0.01$). Plasma TRF contents followed the same rhythm (13 vs. 5 pg/ml) but pituitary and pancreatic TRF did not show any significant rhythm. In female rats we did not find any changes in hypothalamic TRF concentration during the light and dark period.

Although there is no correlation between the hypothalamic content of TRF and plasma TSH level in cold-exposed or thyroid-treated rats (Basahi and Utiger 1972), there seems to be a positive correlation between these variables in relation to the time of day in male

rats according to our present and earlier results. At the moment it is not possible to if pineal and hypothalamic TRF and plasma TSH in male rats are in causal relationship.

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D 54

Diurnal Variation of Hypothalamic LRF Concentration in Female Rats

By J. LEPPÄLUOTO, M. TUOMINEN and H. SELÄNNE, *Department of Physiology, University of Oulu, Finland*

Plasma level of LH in the female rat is a function of the time of estrous cycle and day that highest levels are obtained in the afternoon of proestrus. This would indicate that hypothalamic LRF is secreted before or in the afternoon of proestrus. We have therefore measured hypothalamic LRF concentrations in various times of cycle and day.

Female rats were housed for 2 weeks in a silent room with lights on from 6 AM to 6 PM. Six rats were rapidly decapitated at 4 hrs intervals. Hypothalami around the median eminence and between mamillary bodies and optic chiasma were rapidly excised, extracted with methanol, evaporated, dissolved in buffer and measured in a specific radioimmunoassay for LRF (Seppälä, Ranta and Leppäluoto 1974). Vaginal smears were also taken and studied under light microscope.

Highest median eminence LRF concentrations were found at 4 PM, 794 ± 141 pg LRF per mg of wet tissue in diestrus and lowest at midnight 308 pg/mg ($p < 0.05$ to 4 PM values) and at 4 AM 238 pg/mg ($p < 0.02$) in estrus or in metestrus. Hypothalamic LRF concentrations were significantly lower and varied from 25 to 40 pg/mg without showing any significant diurnal fluctuation.

Low median eminence LRF concentration during the dark period of the day might indicate decreased synthesis of LRF in hypothalamus or increased secretion of LRF from median eminence in the dark. Unaltered LRF concentrations in the rest of the hypothalamus might favour the latter possibility.

GRANT Paolo Foundation.

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Effect of Sympathectomy on Active Transport Mechanisms in Choroid Plexus *In Vitro*

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The choroid plexuses (CP) have at least two main functions: production of cerebrospinal fluid (CSF) and active transport of ions and polar compounds between blood and CSF. The CP are richly supplied with both adrenergic and cholinergic nerve fibres (Edvinsson *et al.*, 1975). Recently sympathectomy was shown to increase CP carbonic anhydrase activity and to enhance CSF production (Edvinsson, *et al.* 1975; Lindvall, Edvinsson and Jönasson, 1977) whereas sympathetic nerve stimulation reduced the CSF formation (Lindvall, Edvinsson and Öwman, 1977). In order to further analyse the physiological role of the sympathetic nerves to the CP the effect of sympathetic denervation was studied on active transport mechanisms in isolated CP.

The uptake of choline (organic base) and of PAH (organic acid) into CP of the lateral ventricle of rabbits was studied *in vitro* at one and two weeks after bilateral and unilateral superior cervical sympathectomy (SE). The CP from the non-operated side from unilaterally denervated animals and CP from non-operated animals served as controls.

The CP tissue-medium ratios (T/M) for choline was increased on the operated side by between 1.25 to 3.75 times one week after unilateral SE. One week after bilateral SE T/M for choline on both sides were of the same magnitude as those of the operated side one week after unilateral SE. SE did not change the uptake of PAH. Furthermore no side to side difference in PAH or choline T/M values was noted one week after preganglionic SE. Two weeks after postganglionic SE. Total and Na⁺/K⁺-activated ATPase activity was not significantly different between the operated and the non-operated side one week after SE.

In conclusion, SE temporarily increases CP uptake of the organic base choline *in vitro* whereas the uptake of PAH and the ATPase activity remained unchanged. Ventricular perfusion experiments may reveal if this reflects a sympathetic regulation of CSF transport mechanisms.

This investigation was supported by the Swedish Medical Research Council (Grant No. 8X-732).

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The Time Course of Cyclic Nucleotide Changes In Anaphylactic Guinea Pig Lung

By A. A. MATHE and R. J. SOHN *Departments of Psychiatry Boston University and Physiology Karolinska Institutet Stockholm Sweden*

The chemical mediators of anaphylaxis affect the level of cyclic nucleotides and, conversely nucleotides influence the release of mediators. Since it has been hypothesized that a fall in cAMP is necessary for the mediators release and that cAMP and cGMP change in opposite directions the time course of these two nucleotides and the release of histamine and PGs immediately following anaphylactic challenge was studied.

Guinea pigs were prepared as previously described (Mathé *et al.* 1975 1977). Following a nose period two schedules were used: 1. the lungs were perfused with Tyrode's and 50 mg ovalbumin injected from 0 to 10 sec; 2. aliquots of minced lungs were placed in nylon mesh bags pre-incubated and then transferred to tubes containing buffer with/without 100 µg/ml ovalbumin. In both schedules after the appropriate time period, lung samples were frozen in liquid N₂. Histamine was determined fluorometrically and PGs and nucleotides by radioimmunoassays.

In perfused lung, anaphylaxis caused an initial cAMP peak, followed by a decrease and a new rise until the end (Table 1). Pattern of cGMP changes was similar to that of cAMP. The nucleotide levels in sensitized lungs did not change with time.

TABLE 1 The time course of changes in cyclic nucleotides in perfused guinea pig lungs during anaphylaxis

Time (sec)	0	10	20	30	40	70	130	250
cAMP	2.1	2.2	5.6**	3.1	1.0*	4.5	7.2	7.9*
cGMP	0.17	0.18	0.20	0.29*	0.10*	0.49**	0.50*	0.73

Results expressed as mean nmol nucleotide/g wet wt. N=5 and * denote P<0.05 and ** denote P<0.01 respectively compared to time 0.

Histamine, PGF_{2α} and 15-keto-13,14-dihydro-metabolite release into the perfusate at 250 sec was 25.1%, 33.3 ng and 167.2 ng respectively (P<0.01 for all mediators).

In minced lung, cAMP peaked at 5 to 10 sec (150 and 136% respectively of time 0, P<0.01) did not differ from baseline at 15, 30 and 45 sec and was several-fold increased at 90, 120 and 240 sec. Changes in cGMP followed a similar pattern. Histamine and PGF_{2α} in the media were elevated from 15 sec (436 and 285% respectively of baseline, P<0.01) and until the end.

The data show that the onset of anaphylaxis results in a rapid and transient increase in cAMP and cGMP. The patterns of cAMP and cGMP changes are parallel. The results further confirm our finding that both nucleotides are elevated in later stages of anaphylaxis (Mathé *et al.* 1974 1977) but do not support the hypotheses that a fall in cAMP is necessary for the release of mediators and that cAMP and cGMP change in opposite directions.

SOVAK, J. and Å. WENNMALM. Influence of indomethacin and of prostaglandin E_2 on total and regional blood flow in man

Communications:

LAURSEN, A. M., B. DYHRE-POULSEN, A. DJØRUP and H. JAHNSEN. Programmed pattern of muscular activity in monkeys landing from a leap

TUVEMO, T. K. STRANDBERG and M. HÅMBERG. Contractile action of a stable prostaglandin endoperoxide analogue on the human umbilical artery

Abstract Supplement:

Abstractum 453 J. HETTA and B. J. MEYERSON. Sexual motivation in the male rat. Ethological study of sex-specific orientation and the effects of gonadal hormones

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In general, a succinct style and restriction to the necessary of documentation and discussion efforts aids in reducing publication time.

References should be given with full title and name of journals, abbreviated in accordance with 4th ed. of *World List of Scientific Periodicals*, with volume number and first and last page numbers.

Figures should not be larger than manuscript pages and sent in as glossy prints in a size larger than required for reproduction. Lettering should be large enough to permit suitable reduction and prefer uniform size. When possible, diagrams and photomicrographs should extend horizontally rather than vertically in order to save space. Photomicrographs should be calibrated on the print (not as enlargement factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum, both in number and size, with text above the table (not on separate sheets). Single numbers in a series should be replaced by mean and S.D. or mean and S.E., in the latter case with number of observations.

Key words (5-10) are recommended in order to facilitate indexing.

For abbreviations, units, and symbols see special list in the Journal and recent articles.

More detailed instructions to authors are under preparation, pending the recommendations of the Scandinavian Publications Committee.

The international system of units (SI)

The following symbols and units, recommended by the SI are being used in *Acta Physiologica Scandinavica*. Certain units, not included in SI, will still be permitted.

SI units with recommended symbols

Units	Symbols
kilogramme	kg
second, millisecond	s ms
mole, millimole, micromole, nanomole, picomole	mol mmol μ mol nmol pmol
meter millimeter micrometer nanometer	m mm μ m nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N ($kg\ m/s^2$)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J ($N\ m$)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m^2)

Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter) (calorie) (kilopond) (millimeters of mercur)	M cal (4.184 J) kp (9.81 N) mm Hg
curie	(1.33 bar)
(millibar)	mbar (100 Pa)
curie	Cl
liter milliliter microliter	l ml μ l
degree Celsius	°C

Conversion factors to be given in Methods.

